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Defining a Role for Inducible Heat Shock Protein 70 (HSP70i) In Mediating Autoimmune Vitiligo

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LOYOLA UNIVERSITY CHICAGO

DEFINING A ROLE FOR INDUCIBLE HEAT SHOCK PROTEIN 70 (HSP70I)
IN MEDIATING AUTOIMMUNE VITILIGO

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY, AND ANATOMY

BY

JEFFREY A. MOSENSON

CHICAGO, IL

DECEMBER 2013
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worked with you. I still have to get our HSP70i T-shirts made up...

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I dedicate this work to my family, the four J’s. I thank you for all of your encouragement and support, and for believing in me no matter what I decided to do in life. And to my three amigos: Mark, Amit and Faisal. I could not have asked for a better group of friends.
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<td>3LL</td>
<td>Murine Lewis Lung carcinoma cells (3LL)</td>
</tr>
<tr>
<td>4-TBP</td>
<td>4-tertiary butyl phenol (4-TBP)</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous lymphoid antigen</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>CTLs</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
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<td>HSPs</td>
<td>Heat shock proteins</td>
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<tr>
<td>HSP70i</td>
<td>Inducible HSP70</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-associated membrane protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MBEH</td>
<td>Monobenzyl ether of hydroquinone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>nMDP</td>
<td>Normalized mean deviation production</td>
</tr>
<tr>
<td>NBUVB</td>
<td>narrowband ultraviolet band</td>
</tr>
<tr>
<td>NLRP1</td>
<td>NLR family, pyrin domain containing 1</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PC-KUS</td>
<td>Pseudocatalase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TRP</td>
<td>Tyrosinase related protein</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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ABSTRACT

Vitiligo is an autoimmune disease characterized by destruction of melanocytes, leaving 0.5% of the population with progressive depigmentation. Current treatments offer limited efficacy. Observations that heat shock proteins (HSPs) can serve as adjuvants in antitumor vaccines first suggested to us a link between HSPs and stress-induced vitiligo. Such proteins are marvelously well conserved throughout evolution, which has placed them in the spotlight for helping to understand the intriguing relationship between infection and immunity. Intracellularly, inducible heat shock protein 70 (HSP70i) is upregulated by stress and helps protect cells from undergoing apoptosis. In times of stress, melanocytes will secrete antigen bound HSP70i to act as an alarm signal in activating dendritic cells (DCs), invoking a targeted response towards melanocytes. Thus HSPs are candidate molecules mediating the transition between causative stress and the autoimmune response to follow. **We hypothesized that HSP70i is important for inducing an autoimmune response in vitiligo through presentation of melanocyte antigens and activation of dendritic cells and, and that blocking its dendritic cell activating region will halt depigmentation.** Data in this dissertation reveal 1) Vitiliginous melanocytes (e.g., melanocytes harvested from vitiligo patient nonlesional skin) secrete higher levels of HSP70i, 2) HSP70i colocalizes with melanocyte antigens, 3) HSP70i is required to induce vitiligo, 4) The...
sequence QPGVLIQVYEG located in the C-terminus is required for immune activity leading to depigmentation, 5) Vaccination with mutant HSP70iQ435A prevents and treats vitiligo by altering antigen presenting cell and T cell profiles. Together these findings suggest that the autoimmune response is funneled through HSP70i, and provide compelling experimental evidence that exposure to inducible heat shock protein 70 (HSP70i) carrying a single amino acid modification offers potent prophylactic and therapeutic opportunities in vitiligo.
CHAPTER I

STATEMENT OF THE PROBLEM

Vitiligo skin lesions are milky-white patches changing in shape and size over time (Ortonne et al., 1983). Once lost, melanocytes are difficult to replace and treatment opportunities are limited, tedious, and largely unsuccessful (Shaffrali and Gawkrodger, 2000). Local, generic immunosuppression by light treatment or corticosteroids are among the most commonly applied remedies. However, the success is limited and involves long lag periods (Grimes, 2004). Several groups have implicated local production of reactive oxygen species (ROS) in vitiligo as well as other autoimmune disorders (Namazi, 2007). To prevent melanocyte death, it has been proposed to increase catalase activity in skin to protect melanocytes; however, this will similarly support longevity of infiltrating T cells (Hultqvist et al., 2007). In particular among dark skinned individuals, the emotional effects can include severe social anxiety, reinforcing the impact of vitiligo on a patient’s quality of life (Mattoo et al., 2002; Sampogna et al., 2004). Thus there is a dire need for treatment measures that can truly inhibit and preferentially reverse depigmentation.

Several theories have been put forth for the cause of vitiligo including: 1) that it is due to a biochemical pathway in which free radicals are formed during melanin production, 2) that it is a disease of neurological origin, or 3) that it is an
autoimmune disorder (Ortonne et al., 1983). The general consensus agrees with the latter hypothesis as there is increasing evidence for autoimmunity as supported by genetic predisposition to candidate genes including MHC class I and II, PTPN22 and IL2RA which are involved with the signaling events in response to antigen; thus, patients with vitiligo have genetic predispositions to other autoimmune disorders (Jin et al., 2010). In addition, skin from vitiligo patients contains increased CTL infiltrates, and patients with vitiligo can frequently develop other autoimmune disorders, in particular thyroid disease (Le Poole et al., 1996; Laberge et al., 2005).

Stress attributed to mechanical trauma or exposure to bleaching phenols has been shown to initiate or intensify skin lesions (Das et al., 2001; Boissy and Manga, 2004; Lerner, 1959). Psychological stress may also contribute to vitiligo etiology (Picardi et al., 2003). Such precipitating factors translate into an autoimmune response specifically targeting melanocytes through unknown mechanisms. Here, we propose a role for heat shock proteins (HSPs) in mediating this response. HSPs are cytoplasmic chaperones upregulated under stress, and are involved in maintaining cellular integrity, and preventing apoptosis (Lindquist and Craig, 1988; Mehlen et al., 1996). Extracellular HSPs in turn function as alarm signals to the immune system by supporting uptake, processing and presentation of antigens in the context of MHC class I and II, and invoking both innate and adaptive responses (Javid et al., 2007; Srivastava, 2002). Dendritic cell (DC) activation then allows for recruitment of CD8+ T cells from draining lymph nodes (Srivastava, 2002).

A potentially unique role of inducible HSP70 (HSP70i) in initiating autoimmunity is better understood considering that HSP70i is actively secreted by
living cells of neuronal origin (Mambula et al., 2007). In fact, melanocytes established from non-lesional vitiligo skin treated with skin bleaching 4-tertiary butyl phenol (4-TBP) show increased secretion of HSP70i; the same stress also confers sensitivity to DC mediated cytotoxicity (Kroll et al., 2005). In addition to active secretion through exosomal release, HSP70i can enter the circulation through non-secretion pathways, such as necrosis (Gallucci et al., 1999; Johnson and Fleshner, 2006). We postulate that under stress, secreted inducible HSP70 serves as an adjuvant in vitiligo, binding melanocytic antigens to be processed by DCs, followed by the breaking of tolerance. HSP70i may activate DCs through specific receptor binding, leading to the production of various cytokines such as IL-12 and IFN-γ, and subsequent activation and recruitment of effector cells from draining lymph nodes (Nicchitta, 2003). Thus, the identification of a peptide within HSP70i crucial to eliciting autoimmunity will be significant as it will open doors to preventing the causative chain of events leading to depigmentation in vitiligo, and autoimmunity in general. This dissertation aims to firmly establish a role for HSP70i in autoimmune vitiligo, identify a targetable peptide within the molecule and importantly, reveal the potentially unique treatment avenue of blocking the effects of HSP70i in activating the immune system.
CHAPTER II: INTRODUCTION

A central role for inducible Heat Shock Protein 70 in autoimmune Vitiligo

(Mosenson et al., Exp. Dermatol., 2013)

Vitiligo is an organ-specific autoimmune disease of the skin.

Vitiligo is a skin disorder presenting with progressive depigmentation and affecting 0.5% of the world population (Krüger and Schallreuter, 2012). Depigmentation is due to the loss of melanocytes from the epidermis (Le Poole et al., 1993). Genetic studies support the involvement of abnormalities affecting immune function in vitiligo (Jin et al., 2010). T cell infiltrates are observed in perilesional skin of patients with active vitiligo (Le Poole et al., 1996). Melanocyte-reactive T cells are relatively abundant among peripheral T cells from patients with active disease (Ogg et al., 1998). T cells isolated from vitiligo skin are cytotoxic towards melanocytes (Wankowicz-Kalinska et al., 2003; van den Boorn et al., 2009). Thus vitiligo is primarily T cell mediated, although humoral responses may also contribute to disease development (Kemp et al., 2011).

Intrinsic abnormalities were found in vitiligo melanocytes, including dilated ER profiles, mitochondrial abnormalities and abnormal melanosome compartmentalization (Boissy et al., 1991), possibly rendering the cells increasingly sensitive to stress (Le Poole et al., 1999). Patients consider stress a precipitating
factor for their disease (Cedercreutz et al., 2010) and known stressors, including bleaching phenols, UV irradiation and mechanical injury invoke a Koebner phenomenon in about half of patients (van Geel et al., 2012). In terms of emotional stress, obsession and phobia has been correlated with autoimmune markers in vitiligo (Moretti et al., 2012). In ‘occupational vitiligo’ individuals develop disease in response to bleaching phenols in the workplace (Boissy and Manga, 2004). These can cause oxidative stress in the skin. Several lines of evidence support an association between oxidative stress and vitiligo (Laddha et al., 2013). This has culminated in the development of pseudocatalase (PC-KUS) treatment for vitiligo, which has unfortunately shown limited efficacy in the clinic (Schallreuter et al., 2008; Bakis-Petsoglou et al., 2009). Gene expression analysis revealed upregulated IL-6 and IL-8 expression by melanocytes in response to bleaching agents (Toosi et al., 2012). Thus stress can cause micro-inflammation and support recruitment of an immune infiltrate to the skin, reflecting the connection between stress and autoimmunity. In summary, vitiligo is a T cell mediated autoimmune disease precipitating under stress.

The immune response presents a mirror image of that found in melanoma.

Antigens recognized by T cells infiltrating vitiligo skin were known targets for T cells infiltrating melanoma tumors (Das et al., 2001). These antigens are predominantly expressed in melanosomes which bear functional resemblance to lysosomes (Raposo and Marks, 2007). Adding a melanosomal trafficking signal enhances immunogenicity of non-melanosomal proteins (Wang et al., 1999; Robila
et al., 2008). Thus, localization likely contributes to the immunogenicity of melanosomal proteins. A resemblance between immune reactivity in vitiligo and melanoma is supported by leukoderma in melanoma patients with detectable immune responses to their tumor. In fact, depigmentation is a positive prognostic factor in melanoma (Quaglino et al., 2010). Unfortunately the immune response rarely clears tumors, whereas robust immunity directed towards the same antigens is a hallmark of vitiligo. A lack of regulatory T cells infiltrating vitiligo skin compared to their abundance in melanoma contributes to such differences (Turk et al., 2004; Klarquist et al., 2010). In fact, autoimmune destruction of melanocytes following Treg depletion in tumor challenged mice is required for generating effective anti-tumor responses (Byrne et al., 2011).

Treatments under development to boost anti-tumor immunity in melanoma include vaccines based on heat shock protein 70 (HSP70) fusion proteins (Qu et al, 2010). HSP70 is included in vaccines as a chaperone protein, immunogenic in its own right (Faure et al., 2007; Stangl et al., 2011) and functioning as an immune adjuvant as described below.

*Inducible HSP70 can mediate immune responses.*

Cells under stress halt mainstream protein synthesis in favor of heat shock protein and/or glucose regulated protein synthesis (Welch, 1993; Määttänen et al., 2012). In the ER this can activate the unfolded protein response (UPR), upregulating heat shock proteins (Gardner et al., 2013). The UPR has been implicated in many diseases including vitiligo (Toosi et al., 2012). This finding is congruent with dilated
ER profiles reported for vitiligo melanocytes, potentially regulated by VIT1/FBXO11 (Le Poole et al., 2001; Guan et al., 2010). Within the cell, stress proteins bind preexisting proteins, promoting autophagy to avert cellular apoptosis (Benbrook and Long, 2012). This function can have implications for vitiligo (Elassiuty et al., 2011). Cell derived stress protein fractions can also ignite immune responses specific to the proteins and peptides they chaperone and thus, to the originating host cells (Calderwood et al., 2012). Among larger heat shock proteins, inducible HSP70 is unique for its secretion from live cells as a chaperokine (e.g., having both chaperone and cytokine activity) (Vega et al., 2008). Other stress proteins likely gain access to the extracellular milieu only after necrotic cell death (Strbo and Podack, 2008). The unique secretory property of HSP70i may be ascribed at least in part to its cellular location, associated in part with melanosomes (Chi et al., 2006). HSP70i is exported by live cells through the endo-lysosomal pathway (Mambula and Calderwood, 2006). A rise in intracellular calcium serves as a signal for exocytosis for several cell types (Johnson and Fleshner, 2006). In this setting DCs are provided with antigenic peptides from live cells for processing and presentation to T cells (Nicchitta, 2003). HSP70i can also stimulate proliferation and cytotoxicity of natural killer (NK) cells (Nicchitta, 2003), and enhance leukotriene secretion by mast cells (Multhoff, 2009; Mortaz et al., 2007). Moreover, HSP70i induces maturation and type-1 polarizing cytokine production by dendritic cells (DCs) and stimulates cross priming of T cells (Krammerer et al., 2012), and importantly, breaks tolerance and induces autoimmune tissue destruction in mice (Millar et al., 2003).
A causative role of HSP70 in autoimmune disease remains controversial (Denman et al., 2008; Wachstein et al., 2012). Most studies identify the C-terminal, substrate binding region of the HSP70 protein as the region dictating immune reactivity, which may in turn be modulated by bound antigen (Wheeler et al., 2011; Spiering et al., 2012). The C-terminus of stress proteins is thus likely important for stimulating DCs (Zhang and Huang, 2006). Subtle sequence differences may define immune activation versus tolerization, as microbial HSP70 was shown to suppress inflammation in several studies (Motta et al., 2007; Borges et al., 2012). The outcome of immune responses was not reported to depend upon the maturation stage of recipient antigen presenting cells. However, the separate identification of immune stimulatory and immune suppressive regions within the C-terminus of HSP70 (Wang et al., 2005) suggests that receptor binding affects the prevailing consequences of HSP70 exposure.

Several surface receptors were implicated in mediating the effects of extracellular HSP70, including CD91, TLR-2, CD14/TLR4, CCR5, and scavenger receptors (Thériault et al., 2006; Aneja et al., 2006; Floto et al., 2006; Qazi et al., 2007; Fischer et al., 2010). Interestingly, elevated surface expression of HSP70 on circulating lymphocytes was reported for vitiligo patients (Frediani et al., 2005). HSP70-induced inflammatory killing of melanocytes confers immunological memory against tumor cells, and may thus enhance autoimmune responses to melanocytes as well (Sanchez-Perez et al., 2006). The ability of HSP70 to chaperone antigenic moieties and to activate a specific, T cell-mediated immune response is exploited in
anti-tumor vaccines (Fletcher et al., 2006; Abkin et al., 2012). Thus HSP70 is a likely contributor to autoimmune reactivity.

**HSP70 is involved in trafficking and degradation of lysosomal proteins.**

The constitutive form of HSP70, HSPA8, reroutes cytosolic proteins otherwise destined for proteasomal degradation to the lysosome (Terlecky, 1994). Proteins rerouted for lysosomal degradation are linearized by a lysosomal membrane complex involving HSP70, then transferred to LAMP-2a molecules forming a pore in the lysosomal membrane (Agarraberas and Dice, 2001). Once inside the lysosome, proteins again encounter HSP70 (lyHSP70) (Agarraberas et al., 1997), to safeguard entering resident lysosomal proteins from inadvertent degradation. In rheumatoid arthritis, autoimmune reactivity was assigned in part to the process whereby HSP70 chaperones proteins into lysosomes (Auger and Roudier, 2005). HSP70 safeguards lysosomal integrity, protecting against conditions of oxidative stress (Nylandsted et al., 2004). When misfolded proteins are no longer remedied by autophagy, loss of lysosomal integrity contributes to programmed necrosis (Yamashima, 2012). Disrupted autophagy may also occur in vitiligo (van den Boorn et al., 2001). Consequently, HSP70 and its co-chaperones (particularly CHIP) appear as gatekeepers defining the proportion of proteins undergoing proteasomal degradation and MHC class I antigen presentation, or lysosomal degradation (Shin et al., 2005). In cells expressing MHC class II molecules, lysosomes are a source of peptides to be presented in the context of such MHC class
II molecules, thus HSP70 helps segregate class I and class II destinations (Zhou et al., 2005; Nedelkovska and Robert, 2013).

Besides professional antigen presenting cells, resident tissue cells can express MHC class II molecules under exceptional circumstances. For melanocytes, these circumstances are met in melanoma, vitiligo and Vogt-Kayanagi-Harada syndrome (Le Poole and Luiten, 2008; Damico et al., 2009). Melanosomes engage in melanosome-endosome fusion and antigen processing (Marks et al., 2003). Mutations in constitutive HSP70 have been implicated in disruption of the endosomal/lysosomal compartment (Chang et al., 2002). Interestingly, overexpression of HSP70i in melanoma cells inhibited melanin production (Hoshino et al., 2010). Overall the presence of HSP70 in melanosomes, potentially involved in trafficking of melanosomal proteins, has not been thoroughly investigated. Yet the exceptional immunogenicity of melanosomes can likely be ascribed in part to melanocyte specific melanosomal proteins presented in the context of MHC class II molecules by melanocytes and melanoma cells (Robila, et al, 2008).

The HSP70's are a complex family of proteins with specific household tasks.

The HSP70 family is composed of at least 17 highly related genes on chromosomes 1, 5, 6, 9, 11, 14 and 21 in humans, encoding constitutively expressed and inducible proteins (Brocchieri et al., 2008). The common denominator is expression induced by elevated temperatures (heat shock) of proteins with an approximate molecular weight of 70 kDa (66-78 kDa) (Tavaria et al., 1996). Three functional domains have been assigned: an N-terminal ATPase domain of
approximately 44 kD (~350 aa), an 18 kD substrate binding domain (~150 aa) and a 10 kD C terminal domain (~100 aa) responsible for binding chaperone cofactors (Lehner et al., 2004). Family members serve as chaperones, guiding intracellular proteins to respective organelle targets (Kriebaumer et al., 2012). In this function HSP70 facilitates folding, binding and translocation of proteins (Gething and Sambrook, 1992). Loci encoding the HSP70 family were named HSPA1 through HSPA14 (Brocchieri et al., 2008). Canonical HSP70 isoforms are functionally redundant, with the main differences found in their spatio-temporal expression (Kabani et al., 2008). The localization of individual gene products will vary from nuclear/cytoplasmic (A1/HSP72/Hsp70i, and A8/ HSP73/HSC70) to ER (5/BiP/GRP78) and mitochondrial (9/GRP75/PBP74) (Tavaria et al., 1996). HSP70 will bind to CD40 by means of its upstream ATPase domain, coinciding with the binding site of chaperone cofactor Hip, stabilizing the ADP state of HSP70 to facilitate peptide binding (Mayer and Bukau, 2005). Substrate specificity may be defined by J protein cofactor binding (Kampinga and Craig, 2010).

A chaperokine function was assigned mainly to inducible HSPA1A (Mambula and Calderwood, 2006). The constitutively expressed isoforms are considered important for cellular housekeeping, whereas inducible isoforms offer protection from stress (Brocchieri et al., 2008; Daugaard et al., 2007). Enhanced secretion of HSP70i by live cells was observed in response to IFN-γ (Bausero et al., 2005), important for vitiligo development (Harris et al., 2012). Gene products protecting cells from the consequences of heat shock are well conserved, and homologues are found across species (Petitjean et al., 2012).
HSP70 is a star player in anti-tumor vaccines and treatment of autoimmune disease.

The chaperone function of HSP70, supporting uptake and processing of antigens by DCs renders the molecule an ideal adjuvant in anti-tumor vaccines (Farzanehpour et al., 2013). DNA encoding HSP70i-antigen fusion proteins was included in vaccines to melanoma (Choi et al., 2011). Such applications frequently use mycobacterial HSP70 (Liu et al., 2009). For anti-cancer vaccines, the use of xenogeneic stress proteins has the added advantage that nucleotide variations render the resulting protein increasingly immunogenic (mycobacterial and mouse HSP70 are approximately 50% homologous), whereas either version can bind peptides and proteins. Meanwhile murine cell lines will bind human HSP70 and vice versa (MacAry et al., 2004).

An intriguing relationship exists between anti-tumor immunity and autoimmunity in melanoma versus vitiligo (Teulings et al., 2013). This ultimately prompted our studies into the involvement of heat shock proteins in vitiligo after HSPs were implicated in anti-tumor immunity (Srivastava and Udono, 1994). Whereas vaccines supporting the role of HSP70 in anti-tumor immunity will benefit melanoma patients, blocking HSP70 from perpetuating autoimmune responses can benefit vitiligo patients. Stress proteins are suited to serve as the ‘molecular funnel’ channeling environmental stress into an autoimmune response targeting melanocytes these events. Initial studies supporting this hypothesis involved studying differential expression of heat shock proteins among non-lesional and lesional vitiligo skin samples (Le Poole and Luiten, 2008). Subsequently, it was shown that heat shock proteins induced DCs to assume a cytotoxic profile, attacking
cells expressing TGF receptor family molecules, including stressed melanocytes (Kroll et al., 2005). Heat shock protein overexpressing melanocytes can support immune response to differentiation antigens (Sanchez-Perez et al., 2006). Overexpression of HSP70i likewise led to accelerated and progressive vitiligo (Denman et al., 2008). As other stress proteins can activate DCs, these may likewise supports vitiligo development.

SUMMARY

Vitiligo is an autoimmune disorder caused by melanocyte-reactive T cells. Importantly, vitiligo is known to initiate and accelerate under stress, thus HSPs are likely candidates in mediating this response. HSP70i plays a pivotal role in the development of autoimmune disorders and activating the immune response in general. Part of this immune activation is thought to occur by DC activating sequences within HSP70i. There is evidence that HSP70i chaperones melanocyte antigens, and may be secreted in response to stress. This peptide chaperoning function may contribute to melanocyte-antigen specific targeting resulting in vitiligo pathology as shown in Figure 1. Taken together, targeting HSP70i offers a novel approach for treating vitiligo.
HYPOTHESIS AND AIMS

A unique feature of HSP70i is that it is actively secreted by living cells. Upon secretion, HSP70 induces immune response by chaperoning antigen, or activating dendritic cells (DC) directly. It is unknown which region(s) of HSP70i activates DCs, whereas multiple HSP70i DC receptors have been identified. Thus identifying the DC stimulatory epitope(s) within HSP70i in itself is novel. I hypothesize that HSP70i is important for inducing an autoimmune response in vitiligo through presentation of melanocyte antigens and activation of dendritic cells, and that blocking its dendritic cell activating region will halt depigmentation. The findings outlined in this dissertation firmly establish a role for HSP70i in vitiligo, identify a targetable peptide within the molecule and importantly, reveal the potentially unique treatment avenue of blocking the effects of HSP70i in activating the immune system.

The following aims were designed to test this hypothesis:

Aim 1: To characterize HSP70i localization and secretion in vitiligo melanocytes

Hypothesis: HSP70i is differentially expressed and secreted in melanocytes harvested from vitiligo skin

Genetic profiling suggests inherent differences in melanocytes between vitiligo and healthy patients (Stromberg et al., 2008). The co-occurrence of stress and vitiligo suggests a role for a molecular pathway to link both processes. Heat shock proteins are prime candidates to initiate disease as they are upregulated in
response to cellular stress, including the inducible isoform of HSP70 (HSP70i) (Morimoto, 1998). These proteins can prevent apoptosis in stressed cells by chaperoning proteins to prevent misfolding (Lindquist and Craig, 1988; Mehlen et al., 1996). The presence of HSP70 on or in melanosomes, potentially involved in trafficking of melanosomal proteins has not been investigated to date. Yet the exceptional immunogenicity of melanosomes can likely be ascribed, at least in part, to melanocyte specific melanosomal proteins presented to the immune system in the context of MHC class II molecules by vitiliginous melanocytes (Wang et al., 1999). Also, the HSP70 associated with melanosomes may be externalized during melanosome transfer, potentially affecting antigen uptake, processing and presentation by DC. In this regard, secretion of HSP70 by live cells appears to be mediated by secretory-like granules (Evdonin et al., 2006). Identifying the localization of HSP70i within melanocytes may reveal an association with melanocyte antigens. Because HSP70i is unique in that it is secreted by live cells and has chaperone activity (Asea, 2007), dendritic cells may be activated after uptaking the peptide bound HSP70i initiating the chain of events leading to an autoimmune response. To date, it is unknown whether HSP70i is differentially expressed or secreted in vitiligo melanocytes. The following sub-aims addressed this question.

a) Compare cell viability of vitiligo and control melanocytes in response to stress
b) Identify subcellular localization of HSP70i within melanocytes
c) Compare HSP70i secretion in vitiligo and control melanocytes
Overall, data from this aim suggests that vitiligo melanocytes are more sensitive to stress (in the form of exposure to bleaching agents) by secreting higher levels of HSP70i. This was shown by performing an HSP70i ELISA on cultured melanocytes from vitiligo and healthy patients. However, these cells are not more prone to cell death as determined by an MTT assay. It was also found that HSP70i is located in the melanosome where it comes into close proximity to melanocyte antigens. This was determined by both confocal and electron microscopy, as well as western blot analysis.

**Aim 2: To assess the requirement of HSP70i for inducing depigmentation in vivo**

_Hypothesis: HSP70i is important for inducing an autoimmune response towards melanocytes via dendritic cell activation and cytotoxic T lymphocyte (CTL) mediated cytotoxicity_

A link between HSP70i and depigmentation was first suggested when inducible murine hsp70 coexpressed with an artificial antigen in mouse melanocytes and melanoma cells resulted in enhanced melanocyte killing (Sanchez-Perez et al., 2006). A direct role for HSP70 in CTL-mediated autoimmunity was then solidified by vaccinating mice with DNA encoding tyrosinase-related protein 2 (TRP-2) in the presence and absence of HSP70i (Denman et al., 2008). These studies demonstrate the importance of inducible HSP70 in breaking CD8+ T cell tolerance towards melanocytes. However, there may be redundancy amongst other HSPs (e.g.
gp96, or HSPs 60, 90, and 100). The following sub-aims addressed the question whether the role of HSP70i is unique and non-redundant, and whether HSP70i is necessary and sufficient to induce vitiligo in order to identify HSP70i as a potential treatment target.

a) Demonstrate whether HSP70i knockout mice are resistant to developing vitiligo
b) Measuring relative DC activation and T cell reactivity to melanocytes
c) Determine if HSP70i is enough to activate autoimmunity in disease-prone mice

From this aim it was determined that HSP70i knockout mice are resistant to developing vitiligo. This was tested by vaccinated C57BL/6 and HSP70i knockout mice with melanocyte antigens. Moreover, it was determined that vaccination with HSP70i alone accelerates depigmentation in vitiligo-prone mice which carry a melanocyte-reactive T-cell receptor transgene expressed by the majority of circulating T cells. Our results showed that vaccination with wild-type HSP70i drives an inflammatory response mediated by activated DCs, and CD8 T cells. Depigmentation was mediated by a CTL response as determined by immunohistochemistry and in vivo CTL assays. Finally we showed that HSP70i activates and drives an inflammatory DC/T cell response by FACS analysis of mouse splenocytes.
Aim 3: To assess HSP70i blocking agents in preventing an autoimmune response

Hypothesis: HSP70i-derivative peptides provide superior immune activation over full length protein and the depigmentation response will be prevented by blocking the putative DC binding region of HSP70i

It is well known that HSP70i activates DCs (Basu et al., 2000; Kuppner et al., 2001); however, no one has identified the stimulatory sequence within mammalian HSP70i. HSP70i may activate DCs through specific receptor binding, leading to the activation and recruitment of effector cells from draining lymph nodes. Thus, the identification of a peptide within HSP70 crucial to eliciting autoimmunity will be significant as it will open doors to preventing the causative chain of events leading to depigmentation in vitiligo, and autoimmunity in general. The amino acid sequence QPSVQIQVYQGEREIAAHNK within the C-terminus of the microbial HSP70 homologue DnaK was previously shown to bind and activate DCs in response to infection (Wang et al., 2005). We thus focused on this sequence to narrow down the region important for inducing an immune response in human HSP70i. Using alignment software, a similar sequence was identified within the sequence QPGVLIQVYGEREIAAHNK, rendering the peptide with the greatest homology as QPGVLIQVYGER (amino acids 435-447) as a relevant candidate involved in DC activation. It was predicted that both the microbial and mammalian sequences would show similar folding patterns based on the evolutionary conservation of the molecule. In this aim, the QPGVLIQVYGER epitope as well as other regions of
HSP70i were rigorously tested for DC binding and immune stimulatory capabilities. We predicted that targeting this region can abrogate this effect in the following sub-aims.

a) Identify the HSP70i region most relevant to autoimmunity
b) Demonstrate involvement of the 13-mer peptide in immune activation
c) Demonstrate whether blocking HSP70i via commercially available antibodies prevents DC activation \textit{in vitro}
d) Test effects of mutant HSP70i in preventing depigmentation and immune responses \textit{in vivo}

Overall, it was determined that the immune activating portion of HSP70i required for vitiligo resides in the C-terminus. Specifically, the DC activating sequence (QPGVLIQVYEG) of HSP70i is required for depigmentation. We showed that mutating the DC activating sequence prevents HSP70i from inducing depigmentation in vaccinated animals, but does not affect overall binding to DCs. To block DC activation by HSP70i, we tested the commercially available HSP70i antibody (SPA-810). It was determined that SPA-810 recognizes a peptide including the sequence PGVLIQVYEG which prevented DC activation \textit{in vitro}, but was unstable \textit{in vivo}. In addition, we discovered that vaccinating vitiligo-prone mice with mutant HSP70i_{Q435A} prevents and treats vitiligo. It was shown that vaccination with HSP70i_{Q435} drives an anti-inflammatory response mediated by decreased (and inhibited) DCs, and the upregulation of anti-inflammatory macrophages. Using immunohistology we showed that wild-type and HSP70i_{Q435} increase and decrease
skin infiltrating CTLs respectively. The mechanism of HSP70iQ435 was mediated by driving quiescent versus T cells as determined by expression of the T cell transcription factors Eomes and T-bet, and through inhibition of DC activation.
CHAPTER III
PREFERENTIAL SECRETION OF INDUCIBLE HSP70
BY VITILIGO MELANOCYTES UNDER STRESS

INTRODUCTION

Vitiligo is a skin disorder characterized by progressive depigmentation. Over 0.5% of the world population is affected by vitiligo, with a 25% increased incidence among women (Cedercreutz et al., 2010). Depigmentation will progress by 1% of body surface area a year on average (Cedercreutz et al., 2010). Expanding lesions are frequently infiltrated by T cells reactive with melanocytes thus supporting an etiologic involvement of cell mediated autoimmunity (van den Wijngaard et al., 2000). Genetic evidence likewise supports a process involving a break in tolerance (Jin et al., 2012), leading to activated T cells trafficking to the skin. However, there is limited concordance between monozygotic twins, suggesting a role for environmental factors in disease precipitation (Spritz, 2010). Indeed, patients appear to develop vitiligo under stressful circumstances (Cedercreutz et al., 2010; Picardi et al., 2003). Stress associated with psychological as well as mechanical/chemical insults can initiate or accelerate vitiligo (Picardi et al., 2003). Several chemicals induce vitiligo, some of which are used in bleaching creams (Boissy and Manga, 2004). These agents may induce oxidative stress, lending
credibility to the observation that antioxidant enzymes such as superoxide
dismutase and catechol-0-methyltransferase are overexpressed in vitiligo skin (Le
Poole et al., 1994; Sravani et al., 2009). Differential gene expression data further
suggest that melanocytes from vitiligo patients may be inherently sensitive to stress
(Stromberg et al., 2008).

To understand why stress would translate into an immune response
specifically targeting melanocytes, it may be important to take note of the natural
target antigens that have been uncovered, which predominantly reside in
melanosomes. These organelles are uniquely immunogenic (Sakai et al., 1997), and
carry lysosome-like properties (Orlow, 1995; Le Poole, 1993a; Raposo et al., 2002).
Moreover, the process of melanogenesis within these organelles is associated with
generating hydrogen peroxide and free radicals during melanogenesis, providing a
direct link to stress-induced disease (Mastore et al., 2005). Thus stress can be a
consequence of the very organelles that form a source of melanocyte antigens.
Taken together with morphologic abnormalities reported for vitiligo melanocytes
also affecting melanosomes (Li et al., 2009; Boissy et al., 1991) it has long been
postulated that patient cells selectively succumb to oxidative stress within vitiligo
skin.

Meanwhile, heat shock proteins (HSPs) are prime candidates to mediate the
process that translates environmental factors and oxidative stress into an
autoimmune disease. Including the inducible isoform of HSP70 (HSP70i), heat shock
proteins are upregulated in response to cellular stress, preventing apoptosis by
chaperoning proteins to prevent misfolding (Beere and Green, 2001). When found
in the extracellular milieu, stress proteins serve as an alarm signal to the immune system by activating local antigen presenting cells (APCs) (Kammerer et al., 2002). HSP70i is unique in this regard, as it is reportedly secreted by live cells (Asea, 2007).

Several lines of evidence have since solidified a role for HSP70i in precipitating vitiligo, including the observation that wild-type and vitiligo-prone mice develop accelerated disease in response to HSP70i (Mosenson et al., 2012; Mosenson et al., 2013). It was also determined that mice lacking the HSP70i gene do not depigment, attesting to a requirement for the HSP in disease development (Mosenson et al., 2012). The importance of HSP70i in initiating vitiligo may be attributed to its potent adjuvant and chaperone properties (Zhang et al., 2006). This is supported by prior studies which have shown that HSP70i can chaperone immunogenic peptides derived from melanocyte differentiation antigens (Noessner et al., 2002).

Here we address the hypothesis that disease precipitation in vitiligo follows a process whereby HSP70i is in contact with immunogenic melanosomal peptides and preferentially exported from vitiligo melanocytes under stress. We thus exposed primary melanocytes to phenolic agents modeling factors that can precipitate disease, comparing the sensitivity of cells from vitiligo and healthy origin in viability assays. Colocalization of HSP70i and melanosomal proteins was tested by Western blotting of fractionated cells, accompanied by electron microscopic analysis of concentrated fractions. Differential melanosome trafficking of HSP70i in vitiligo melanocytes after treatment with phenolic agents was followed by confocal microscopy using software plugins, while stress protein secretion was measured by
ELISA. Taken together, the experiments can help to understand the interplay between melanocytes and the environment to precipitate disease.
RESULTS

HSP70i is aberrantly expressed in vitiligo skin

Vitiligo is frequently associated with stress which may be accompanied by expression of HSP70i. We thus probed nonlesional, perilesional and lesional skin sections from four vitiligo patients with the HSP70i specific antibody SPA-811 (Figure 2A). HSP70i expression was observed mainly in the epidermis, with highest expression in the lesional sections. Lesional skin contained intense immunoperoxidase staining of all cells and extracellular matrix, whereas nonlesional skin was mostly devoid of HSP70i expression (Figure 2A). Due to these staining patterns, HSP70i expression was evaluated by blinded subjective quantification of immunoperoxidase intensity. It was determined that HSP70i expression is significantly higher in lesional ($P = 0.0044$) and perilesional ($P = 0.0480$) versus nonlesional skin (Figure 2B). These data support previous findings that HSP70 is overexpressed in vitiligo skin, specifically the inducible isoform.
Figure 2. HSP70i overexpression in vitiligo skin. (A) Representative immunoperoxidase staining of HSP70i in vitiligo skin displaying expression predominantly located in the epidermis, with minimal cellular expression observed in nonlesional (left), and moderate to strong expression in perilesional (middle) and lesional (right) skin. (B) Subjective, blinded quantification of HSP70i expression in tissue sections. Immunoperoxidase intensity: 1=low, 2=medium, 3=high. Data are presented as mean ± SEM. Student's one-tailed t-test. *P<0.05, **P<0.01, n=7 subjects.
**Vitiligo and control melanocytes are equally sensitive to stress induced by phenolic agents**

The above data suggested that the overexpression of HSP70i in vitiligo skin may play a protective role in response to stress. Thus, we measured the viability of primary melanocytes harvested from nonlesional vitiligo and control skin in response to the phenolic agents 4-tertiary butyl phenol (4-TBP) and monobenzyl ether of hydroquinone (MBEH). The melanocytes were exposed to 125, 250, and 500 μM of 4-TBP or MBEH for 72 hours, and viability was determined by MTT assay (Figure 3). All samples were compared relative to vehicle (20% dimethyl sulfoxide diluted in 70% EtOH, and diluted to 1:1000 in melanocyte media) treated control cells. Treatment with 4-TBP reduced viability by approximately 4, 24, and 69% in response to 125, 250, and 500 μM concentrations of 4-TBP respectively (Figure 3). Treatment with MBEH reduced viability by approximately 19, 32, and 62% in response to 125, 250, and 500 μM concentrations of MBEH respectively (Figure 3). Our results indicate that melanocyte viability decreases as the concentration of 4-TBP and MBEH increases; however, there were no differences between control and vitiligo melanocytes in response to any of the treatments (Figure 3). These data were performed in duplicate with similar results, and demonstrate that vitiliginous melanocytes are not more resistant or susceptible to direct killing by bleaching agents.
Figure 3. Cell viability of vitiliginous melanocytes. Primary melanocytes from control and vitiligo patients were plated at 10,000 cells per well in triplicate, and exposed to 125, 250, or 500 μM concentration of 4-tertiary butyl phenol (4-TBP) or monobenzyl ether of hydroquinone (MBEH) for 72 hours. Percentage viability was quantified in MTT assays, and compared relative to vehicle (20% dimethyl sulfoxide diluted in 70% EtOH, and diluted to 1:1000 in melanocyte media) treated controls. Cell viability decreased as 4-TBP and MBEH dosage increased; however, no differences were observed between control and vitiligo melanocytes for any treatment. Data provided from two independent experiments with triplicate values for each experiment, with similar results. Healthy control (n =8 subjects), vitiligo (n =5 individual cultures). Data are presented as mean ± SEM. Student’s one-tailed t-test.
Vitiligo melanocytes specifically increase HSP70i colocalizing within the melanosome fractions in response to stress

To assess HSP70i and melanosomal antigen colocalization, melanocyte homogenates were fractionated by density gradient centrifugation (Figure 4A), and proteins within individual fractions were further separated by SDS-PAGE (Figure 4B). Blotted proteins were probed with the pAb SPA-820 which binds both the constitutive and inducible HSP70 isoforms. Intense immunoperoxidase detection (40.56% mean band intensity) of HSP70 by SPA-820 was observed in the melanosomal fractions (19-24) whereas weak or no HSP70 expression was detected (6.33% mean band intensity) in the non-melanosomal fractions (1-18) (Figure 4C). Fractions 7-9 and 20-22 were pooled, concentrated and processed for transmission electron microscopy (Figure 4B). Electron micrographs of fractions 20-22 support a high concentration of late melanosomes, coinciding with high content of HSP70. In order to delineate the presence of inducible HSP70 amongst these fractions, blotted proteins were probed with the HSP70i specific mAb SPA-810. Again, detection of HSP70i was limited to the melanosomal fractions (19-24; Figure 4D). In addition, the presence of the melanosomal antigen gp100 (Ab HMB45) was detected selectively amongst these fractions (Figure 4D). These data indicate that HSP70i is in close proximity to melanocyte antigens.
Figure 4. HSP70i colocalizes with melanosomal fractions. (A) Adult melanocytes (HM162P7) were dounced and underloaded on an iodixanol gradient for ultracentrifugation. Dense bands containing melanin were observed in the melanosomal (20/21 and 23) fractions. (B, upper image) Collected fractions (non-melanosomal 7-9, and melanosomal 20-22) were fixed and analyzed by electron microscopy (EM). (B, lower image) Collected fractions were run on a Western Blot. SPA-820 antibody (which binds both constitutive and inducible HSP70 isoforms) reacted with the melanosomal (19-24) but not non-melanosomal fractions (1-15). (C) Quantification of western blot band intensities shown in EM. Mean luminosities indicate stronger antibody reactivity in fractions 19-24 versus 1-18. Student’s one-tailed t-test, * P<0.05. (D) Western blot analysis of non-melanosomal (1, 2 and 18) and melanosomal (19-23) fractions probed with the antibody SPA-810 which only detects inducible HSP70 (70 kD), and HMB45 to the melanocyte antigen gp100 (HMB45 detects a 45 kD product of gp100). Only the melanosomal fractions (19-23) react with both HSP70i and gp100 antibodies. Purified HSP70i protein was probed as a control. Together these data indicate that HSP70i is expressed within the melanosome containing fractions.
Intracellular HSP70i colocalizes in part with melanosomal proteins

To further examine the location of HSP70i within melanocytes at rest, fluorescently labeled antibodies to HSP70i (GFP) and TRP-1 (PE/Cy7) were detected by confocal microscopy (Figure 5). Images of 1.0 μm serial Z-slices indicated that both HSP70i and TRP-1 are ubiquitously expressed throughout the cytoplasm, but not in the nucleus (Figure 5A). HSP70i appears to have a punctate pattern directly outside the nucleus, whereas TRP-1 is mainly detected within the dendrites (Figure 5A). Next, primary vitiligo and control melanocytes were exposed to sub-lethal doses (125 μm) of 4-TBP or MBEH for 72 hours. We analyzed HSP70i (GFP) and the melanocyte antigen gp100 (PE/Cy7). The gp100 reactive antibody HMB45 was selected as it was previously shown to bind a 35 kD fragment of gp100 located predominantly in the melanosome (Harper et al., 2008). The confocal images displayed colocalization of HSP70i and gp100 as shown by Z-slices and merged images (Figure 5B). Interestingly, punctate staining of HSP70i was detected outside the melanocytes suggestive of secretion (Figure 5B). Extracellular gp100 staining was also visible to a lesser extent, possibly due to degradation or loss of the HMB45 binding epitope (Figure 5B). No extracellular staining was observed in the control slides where no primary antibody was added (Figure 6). Colocalization of HSP70i and gp100 was quantified using the ImageJ colocalization plug-in JACoP (Just Another Co-localization Plugin; Bolte and Cordelières, 2006), which calculates the distance between fluorescent pixels (green and red), and also assigns a pseudocolor representing gradation of colocalization (Figure 5B). The number of HSP70i/gp100 colocalized pixels was compared relative to the total number of HSP70i labeled
pixels within the cell. The data indicate no difference in the amount of HSP70i and gp100 colocalization in vitiligo and healthy melanocytes treated with vehicle (Figure 5C). When treated with bleaching phenols, however, the amount of HSP70i and gp100 colocalization differed between vitiligo and healthy melanocytes. Importantly, vitiligo melanocytes treated with MBEH demonstrated the greatest colocalization (7.5%) as compared to any treatment (Figure 5C; \( P = 0.0001 \)). Vitiligo melanocytes treated with 4-TBP displayed ~2-fold increased HSP70i/gp100 colocalization as compared to healthy melanocytes (Figure 5C; \( P = 0.0439 \)). However, less colocalization was detected in melanocytes after treatment with 4-TBP compared to vehicle (Figure 5C). This prompted us to further assess HSP70i expression in response to 4-TBP. Healthy melanocytes were exposed to 125 um 4-TBP for four hours, followed by immunostaining with gold particle-conjugated HSP70i antibodies. Detection was performed by transmission electron microscopy (Figure 7). The images display HSP70i (gold particles) throughout the cytoplasm, which are more abundant in the 4-TBP treated melanocytes (Figure 7). Labeling was quantitated as described in Materials & Methods. The expression of HSP70i in control and 4-TBP treated melanocytes was 10.1 (± 4 SD) and 25.2 (± 3 SD) gold particles/50x10^{-6} \text{ nm}^2, respectively (\( P=0.0002 \)). HSP70i juxtapositioned to melanosomes could be observed under higher magnification (Figure 7, insets). Together with the confocal images, these data demonstrate that HSP70i traffics to the melanosome which can be significantly enhanced in vitiligo melanocytes exposed to MBEH.
Figure 5. HSP70i is overexpressed in vitiliginous melanosomes after stress. (A) Representative serial 1.0 μM Z-sliced images of neonatal melanocytes (Mf0627P11) indicate cytoplasmic expression of HSP70i (SPA-811 Ab detected by FITC) and the melanocyte antigen TRP-1 (Ta99 Ab detected by PE/Cy7) throughout the cell, but not present in the nucleus (DAPI counterstained). (B) Representative 0.5 μM Z-slice images of neonatal melanocytes (Mf0627P11) probed with antibodies to HSP70i (SPA-811) and gp100 (HMB45). Individual channels for HSP70i (GFP), gp100 (PE/Cy7) and merged images are shown. Note extracellular detection of HSP70i/gp100. An image pseudocolored by the ImageJ plug-in JACoP indicates levels of HSP70i/gp100 colocalization as low (blue), middle (yellow) and high (red). Perinuclear red mapping indicates over-lapping between the two labels, suggestive of colocalization. (C) Vitiligo and neonatal melanocytes were treated with 250 μM bleaching agents (4-TBP and MBEH) for 24 hours followed by confocal microscopy. Five z-slices from representative treated melanocytes were analyzed for HSP70i/gp100 colocalization using JACoP, with an nMDP cutoff of 1 used in the calculations. Data is presented as HSP70i/gp100 colocalization relative to total HSP70i staining. Graphed is the normalized mean deviation production (nMDP) for 5, 1μm Z-slices for each sample. Healthy control (n =3 individual cultures), vitiligo (n =3 individual cultures). Data are presented as mean ± SEM. Student’s one-tailed t-test.* P<0.05, ***P< 0.001
Figure 6. Minimal background in confocal no primary images. Representative 0.5 μM Z-slice control images of a neonatal melanocyte (Mf0627P11) probed with anti-rabbit FITC and anti-mouse PE/Cy7 antibodies, excluding primary antibodies. A brightfield image displays the location of the melanocyte, with its nucleus visible in the DAPI channel. Minimal background fluorescence is observed in the FITC and PE/Cy7 channels, demonstrating the secreted HSP70i/gp100 (Figure 5) is not an artifact.
Figure 7. Transmission electron microscope detection of HSP70i in 4-TBP treated melanocytes. Healthy melanocytes (Mf06321P2) were treated with 125 μM 4-TBP for 4 hours followed by immunostaining with gold particle-conjugated antibodies to HSP70i. The melanocytes were next fixed and scanned by transmission electron microscopy. Gold particles (red arrows) are observed in sections probed with anti-HSP70i which are more abundant ($P < 0.0002$, $n=8$ micrographs) in 4-TBP treated melanocytes. HSP70i is seen throughout the cytoplasm in line with data shown in Figure 5, and occasionally justapositioned to melanosomes (insets). Bar equals 500 nm. (inset bars equal 40nm). Student’s one-tailed $t$-test.
Primary vitiligo melanocytes secrete more HSP70i in response to MBEH treatment

The above data support that HSP70i is in close proximity to melanocyte antigens in cells under stress. Extracellular HSP70i is known to have adjuvant properties, thus we next compared HSP70i secretion of vitiligo and control melanocytes in response to chemical stress. It was previously published that immortalized vitiligo PIG3V cells secrete more HSP70i in response to the bleaching agent 4-TBP versus immortalized control PIG1 cells (Kroll et al., 2005). The above confocal data suggested that MBEH greatly upregulates HSP70i trafficking to melanosomes, therefore we investigated its effects on HSP70i secretion. Here, we exposed the same immortalized PIG1 and PIG3V cultures, representing control versus vitiligo melanocytes respectively, to MBEH. The PIG1 and PIG3V cells, as well as primary melanocytes from healthy and vitiligo patients were treated with 125 um of MBEH for 24 hours. Images indicate that exposure to a low dose of MBEH does not induce significant cell death in either the immortalized or primary cell lines (Figure 8A and B), similar to the results in Figure 2. The supernatants were then analyzed for HSP70i by high sensitivity ELISA, and measurements were compared relative to untreated samples. The results confirm that PIG1 and PIG3V cells secrete ~ 3-fold increased levels of HSP70i after MBEH treatment, comparable to reported findings using 4-TBP (Kroll et al., 2005). Importantly, PIG3V cells secrete significantly higher levels of HSP70i as compared to PIG1 cells with vehicle ($P = 0.0145$) and MBEH ($P = 0.0053$) treatment (Figure 8A). In addition, it was revealed that primary vitiligo melanocytes secrete ~7-fold increased amounts of HSP70i after MBEH versus vehicle treatment, whereas no detectable levels of HSP70i were
observed with healthy melanocytes (Figure 8B). HSP70i secretion was similar between vitiligo and healthy melanocytes after vehicle treatment (Figure 8B). Most importantly, MBEH induced significantly greater HSP70i secretion in vitiligo melanocytes \( (P = 0.0031; \text{Figure 8B}) \). In total, these data indicate that vitiligo melanocytes respond to stress by secreting higher levels of HSP70i after phenol exposure.
Figure 8. Vitiligo melanocytes secrete more HSP70i in response to MBEH. (A) The immortalized melanocyte lines (vitiligo PIG3VP54 and control PIG1P96; n=3 measurements) and (B) primary healthy and vitiligo melanocytes were treated with 125 um MBEH for 24 hours. Supernatants from treated and untreated cultures were assessed for HSP70i content by high-sensitivity ELISA. The percent increase in HSP70i of MBEH and vehicle treated compared to untreated samples is indicated. Images of cell cultures (A and B) show no differences in cell density or death after MBEH treatment. A significant increase in HSP70i secretion was observed in vitiligo, but not control melanocytes after MBEH treatment. These data indicate that melanocytes obtained from vitiligo skin secrete more HSP70i in response to stress than healthy cells. Control (n =4 individual cultures), vitiligo (n =3 individual cultures). Data are presented as mean ± SEM. Student’s one-tailed t-test. **P < 0.01, ***P < 0.001.
DISCUSSION

Controversies remain regarding the etiology of vitiligo. There is support for an overproduction of free radicals in stressed vitiligo melanocytes, as well as for an autoimmune response wherein T cells specifically target melanocytic antigens. The data presented in this manuscript suggest that the above may not represent mutually exclusive mechanisms, but rather aspects of disease that complement each other, joining forces to initiate and perpetuate vitiligo development. This supports the convergence and melanocytorrhagy theories which state that autoimmunity, impaired redox status, and genetic predisposition all contribute to vitiligo development (Le Poole et al., 1993b; Kumar and Parsad, 2012).

Melanocytes obtained from vitiligo patients express intrinsic abnormalities including dilated endoplasmic reticulum profiles and abnormal melanosome compartmentalization (Li et al., 2009; Boissy et al., 1991). Moreover, others have reported that melanocytes from vitiligo patients have reduced calcium uptake, which is important for inhibiting redox activity (Schallreuter-Wood et al., 1996). Such observations suggest that vitiligo melanocytes are more sensitive to stress, and generate hydrogen peroxide and other free radicals, especially during melanogenesis. Increased production of H$_2$O$_2$ may increase the susceptibility of melanocytes to apoptosis/necrosis (Zhang et al., 2013). Although a preliminary study suggested that vitiligo melanocytes may be more sensitive to 4-TBP than control cells, the most important data from that study is the restoration of cell viability in presence of catalase (Manga et al., 2006). Meanwhile, the sensitivity of cells to bleaching phenols is greatly affected by donor pigmentation, donor age and
culture conditions, and a comparison among multiple cultures is required to identify overall differences in viability upon exposure. In our current study, the data indicate that cell viability of vitiligo melanocytes is not different from that of healthy melanocytes in response to bleaching agents. Thus, the loss of melanocytes from vitiligo skin likely involves a different pathway. This prompted us to identify a factor that translates stress into melanocyte death observed in vitiligo.

Patient surveys indicate that vitiligo is initiated and accelerated after stress, with ~50% of patients reporting a Koebner phenomenon (Cedercreutz et al., 2010). Heat shock proteins are produced in response to stress, and function to re-fold damaged proteins to prevent cell death (Beere and Greene, 2001). Recently, it was shown that HSP70 is overexpressed in lesional and perilesional vitiligo skin; however, it was unclear which isoform(s) of HSP70 is affected as antibody W27 used in this study was directed to both the constitutive and inducible isoforms (Abdou et al., 2013). As inducible form of HSP70 (HSP70i) is the primary instigator of immune responses (Mosenson et al., 2021), this prompted us to focus on the inducible form of HSP70 (HSP70i) as a mediator of vitiligo. Here we determined that inducible HSP70 specifically is overexpressed in vitiligo lesional tissue. This corresponds with our recently published data that HSP70i is a critical component in vitiligo development (Denman et al., 2008; Mosenson et al., 2012). Besides activating dendritic cells (DCs) to become lytic towards tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor expressing melanocytes, it was shown that HSP70i overexpression can enhance vitiligo development in mice (Kroll et al., 2005; Mosenson et al., 2012). Moreover, a central role for HSP70i was
determined by demonstrating that Hsp70-1 knockout mice are resistant to depigmentation after vaccination with melanocyte antigens (Mosenson et al., 2012).

We previously reported that vitiliginous melanocytes overproduce HSP70i in response to 4-TBP induced stress (Kroll et al., 2005). This appears counterintuitive since HSPs protect cells from apoptosis under stress. However, extracellular HSP70i serves as an “alarm signal” to activate immune responses (Kammerer et al., 2002). This illustrates the importance of HSP70i in immune activation as it is the only HSP secreted by live cells (Asea, 2007). This suggests that immune targeting follows stress to melanocytes as a factor in vitiligo mediated by HSP70i secretion. Here, we predicted that HSP70i would be differentially secreted by MBEH treated control and vitiliginous melanocytes. Using a high sensitivity ELISA we demonstrated that immortalized PIG3V vitiligo melanocytes show relatively increased HSP70i secretion compared to PIG1 immortalized normal melanocytes upon MBEH exposure, similar to data previously using 4-TBP (Kroll et al., 2005). Importantly, we also established that HSP70i is more abundantly secreted by primary vitiliginous melanocytes. These data suggest that vitiliginous melanocytes respond to stress by overproduction/secretion of HSP70i rather than by direct cell death. We propose that the increased HSP70i secretion by vitiligo melanocytes is subsequently followed by selective immune activation and recruitment of melanocyte-reactive cytotoxic T lymphocytes (CTLs), which ultimately contribute to vitiligo development.

HSP70i binds proteins/peptides to serve many functions including protein folding, trafficking, and potentially MHCI/II loading (Srivastava, 2002). Within
melanocytes, melanosomal proteins such as TRP-1 and gp100 are transported from endosomes to lysosomes by HSP70i to be transferred to LAMP-2a proteins for internalization (Terlecky, 1994; Agarraberes and Dice, 2001). Intriguingly, vitiliginous melanocytes express MHCII which is normally reserved to APCs (Le Poole et al., 1993c). Thus, melanocytes under stress may upregulate expression of HSP70i which cross-presents melanocyte-antigens to MHCII as has been observed in other cell types (Srivastava, 2002).

Besides these intracellular immune functions, HSP70i residing in melanosomes may also be externalized during melanosome transfer, potentially affecting antigen uptake, processing and presentation by DCs. Secretion of HSP70i by live cells has been recently suggested to involve lysosomal contents, and the formation of exosomes, and appears to be mediated by secretory-like granules (Evdonin et al., 2006). In melanoma, under minimal stress, low levels of HSP70i are transported to the membrane via endosomes, whereas under stress, elevated levels of expression lead to re-routing of HSP70i into lysosomes for subsequent secretion (Juhasz et al., 2013). Extracellular HSP70i can induce DCs to more actively phagocytize and cross present antigen, and activate responder T cells (Srivastava, 2002), thus the peptides bound to HSP70i may invoke a targeted immune response. Because of these adjuvant properties, HSP70i has been useful in melanoma vaccines (Zhang et al., 2006).

Stress proteins isolated from melanoma tumors will chaperone melanocyte-specific peptides critical for directing a targeted response towards these antigens (Noessner et al., 2002). This antigen-bound HSP70i can activate DCs leading to
production and expansion of tumor reactive T cells. Not surprisingly, T cells isolated from both melanoma and vitiligo patients recognize many shared melanocyte antigens. Confocal microscopic analysis shows that a fraction of intracellular HSP70i is associated with melanosomes. The same is shown by electron microscopic and Western blot analysis of fractionated cells. This follows prior observations that constitutive HSP70 is responsible for shuttling lysosomal content across the organelle membrane (Agarraberes and Dice, 2001). Given the similarity in function of melanosomes within melanocytes and lysosomes in other cells (Orlow, 1995; Le Poole et al., 1993a; Raposo et al., 2002), a shuttle function for the other isoform may explain the melanosomal association and its observed increase under stress.

HSP70i can bind melanocyte antigens (Noessner et al., 2002), and can be found in close proximity to relevant target antigens. Interestingly, we showed that HSP70i colocalizes with melanosomes to a greater extent after MBEH exposure. Thus stress likely induces trafficking of HSP70i to the melanosome where it may assist in protein folding, or antigen loading (Chi et al., 2006). This is coherent with the observation that a melanosome transport signal enhances immunogenicity of intracellular proteins (Wang et al., 1999). Greater colocalization occurred in response to MBEH, whereas 4-TBP gave little to no effect, supporting the very different responses of melanocytes to either form of stress (Hariharan et al., 2011). This is also in line with evidence that MBEH and 4-TBP induce melanocyte cell death by different mechanisms (Hariharan et al., 2011).

Overall, our data indicate that vitiliginous melanocytes are indirectly more sensitive to MBEH-induced stress though increased secretion of HSP70i and
subsequent immune activation. MBEH and 4-TBP are known to induce reactive oxygen species (ROS) (van den Boorn et al., 2011; Manga et al., 2006), which will contribute to HSP70i production (Kim et al., 2005). Once upregulated, HSP70i traffics to the melanosome where it can bind antigenic peptides (Noessner et al., 2002). Melanosomes contain MHCI/II in addition to several melanocyte antigens which can be presented (Wang et al., 1999). Although the mechanism of HSP70i secretion is not well understood, recent evidence supports an exosomal mechanism (Lancaster and Febbraio, 2005) that involves melanosome occlusions (Ando et al., 2012). Within the cell HSP70i may contribute to MHCI/II antigen loading and membrane transport (DeNagel and Pierce, 1992; Castelli et al., 2001). Upon secretion, the melanocyte-antigen bound HSP70i can activate DCs leading to targeted killing of melanocytes. Our data provide a mechanism by which stress differentially induces overexpression and melanosome localization of HSP70i within vitiligo melanocytes, leading to enhanced secretion of HSP70i-antigen complexes, subsequent DC activation and T cell recruitment. Thus stress may selectively initiate an autoimmune response in vitiligo patients which leads to melanocyte cytotoxic T lymphocyte killing.
CHAPTER IV

HSP70i is a critical component of the immune response leading to autoimmune vitiligo (Mosenson et al., Pigment Cell Melanoma Res, 2012)

INTRODUCTION

Vitiligo is a T-cell mediated autoimmune disease of the skin. Patients present with progressive depigmentation that advances at an average rate of 1% bodily surface area a year (Cedercreutz et al., 2010). Genetic predisposition has been assigned to candidate genes including MHC classes I and II, PTPN22 and IL2RA, supporting the autoimmune etiology of the disease (Jin et al., 2010). Approximately 25% more women than men develop vitiligo (Cedercreutz et al., 2010). At this time, there is no definitive evidence to support an association between vitiligo and a particular ethnic background. However, the progressively depigmenting skin is clearly more apparent for patients with a darker skin tone. Also, the social implications of disease are particularly devastating in countries where leprosy is a considerable health problem, as the appearance of skin lesions can bare similarities among both patient groups, and patients with vitiligo are mistakenly identified as individuals expressing a contagious disease (Millington and Levell, 2007).

Aside from a hereditary component, vitiligo pathogenesis involves environmental factors that contribute to precipitating the disease. Among these
exposure to skin bleaching phenols has been particularly well studied (Boissy and Manga, 2004). It is of great interest to identify the molecular connection between environmental precipitating factors and the autoimmune response that follows, as such studies can serve to identify candidate target molecules to develop novel and effective treatment strategies for the disease. There is a lack of effective therapeutics available for vitiligo, in part because halting disease progression and re-establishing pigmentation are conceptually different phases of the disease that may have to be addressed by separate means (Le Poole and Luiten, 2008).

Establishing pigmentation in patients with active disease is likewise problematic. An important step forward in developing vitiligo therapeutics is thus to recognize that progressive disease must first be effectively brought to a halt. Understanding of mechanisms at work in progressive disease will be important. In this respect, heat-shock and glucose-regulated proteins are prime candidates to connect stress to the skin with the autoimmune response to follow. In particular, gp96 and HSPs 60, 70, and 90 have been implicated in immune cell activation (Multhoff, 2006). Stress proteins support immune reactivity by activating dendritic cells to more efficiently phagocytize, process, and present antigens (Murshid et al., 2008). This concept has been elaborately exploited in the design of anti-tumor vaccines for melanoma, renal cell cancer, and less immunogenic tumor types (Tosti et al., 2009).

Among heat-shock proteins, inducible HSP70 (HSP70i) stands out as it can be secreted by live cells (Asea, 2007). Secretion of HSP70i by live cells would thus indicate that even in the absence of cell death, an immune response can be triggered
to proteins and peptides derived from the cells under stress, chaperoned by HSP70i. This has guided an interest in defining the involvement of HSP70i in autoimmune vitiligo. Interestingly, heat-shock proteins have been implicated in other autoimmune diseases as well, including rheumatoid arthritis, and other skin disorders such as psoriasis (Millar and Ohashi, 2007).

After observing consistent differential expression of HSP70i in non-lesional and vitiligo skin from three patients (Le Poole and Luiten, 2008), the concept was tested that the expression of inducible HSP70 is a major contributor to the development of vitiligo in mice. Accelerated depigmentation was associated with enhanced cytotoxic T-lymphocyte (CTL) responses to the vaccinated antigen, supporting that depigmentation occurs in a T-cell mediated fashion in a vaccine-based model of vitiligo (Denman et al., 2008). Also, melanocyte-directed overexpression of HSP70 can enhance anti-tumor responses to melanoma (Sanchez-Perez et al., 2006).

Whereas HSP70i can induce autoimmune depigmentation, given the shared chaperone and immune-activating functions, the same is likely to hold true for other stress proteins as well. The question then remains whether a single stress protein can be held responsible for mediating progressive disease or whether there is redundancy in this mechanism. The HSP70 family of proteins is extensive, with estimates of up to 17 genes in human (Kabani and Martineau, 2008) and 3 in mouse (Snoek et al., 1993). The canonical HSP70 isoforms are functionally redundant, with the main differences found in their spatio-temporal expression (Kabani and Martineau, 2008). The constitutively expressed isoforms are considered important
for cellular housekeeping, whereas the inducible isoforms protect the cell from stressful conditions (Daugaard et al., 2007; Kabani and Martineau, 2008).

What remains to be understood is whether HSP70i is critically important to vitiligo development. If the stress protein is redundant, we expect that in the absence of HSP70i another stress protein can mediate depigmentation. This issue requires resolution before considering HSP70i depletion in the treatment of vitiligo. Here, we addressed whether HSP70i is solely responsible for vitiligo pathogenesis using different animal models. Initially, HSP70 knockout mice were vaccinated with DNA encoding melanocyte-specific target antigens and assessed for progressive depigmentation, melanocyte loss, T-cell infiltration, and CTL activity. These experiments were performed in Hsp70-1 knockout animals lacking inducible HSP70 (Hunt et al., 2004) and repeated with Hsp70-2 knockout animals that lack a constitutively expressed, tissue-specific isoform of the protein important for spermatogenesis (Eddy, 1998). Next, a vitiligo-prone model was used to analyze whether HSP70i alone is sufficient to support progressive depigmentation. This model established by the group of N. Restifo at NIH carries a gp100-reactive T-cell receptor transgene that is expressed by the majority of circulating T cells (Overwijk et al., 2003). Untreated animals develop minor depigmentation by approximately 9 months of age. Finally, blocking of HSP70i-induced Dendritic cell (DC) activation by antibodies was assessed using bone marrow-derived dendritic cells. Taken together, these experiments provide a solid mean to identify HSP70i as a potential target for immunotherapy of vitiligo.
RESULTS

*HSP70i is uniquely required for autoimmune depigmentation*

To evaluate redundancy of the involvement of HSP70i in depigmentation, wild-type C57BL/6 and inducible hsp70 knockout (Hsp70-1) mice were gene gun vaccinated with antigenic mouse Tyrp1ee (optimized TRP-1)-encoding plasmid, an antigen more potent than TRP-2, to ensure that even the most subtle depigmentation responses may be detectable (Guevara-Patino et al., 2006). Gene gun vaccinations involve coating gold particles with gene-encoding plasmids, which are then rapidly fired into the skin via pressurized helium. Four weeks after the final vaccination, we observed that depigmentation was virtually absent in the Hsp70-1 knockout mice (Figure 9A) and confirmed this using image analysis (Figure 9B). By contrast, 16.9% depigmentation was detected in wild-type mice at the vaccination site. Including the human HSP70i-encoding plasmid in the vaccine is insufficient to restore the depigmentation process in Hsp70-1 knockout mice and resulted in 23.9% depigmentation in wild-type mice at the vaccination site (Figure 9A, B). We also followed depigmentation in Hsp70-2 knockout mice gene gun vaccinated with DNA encoding human TRP-2 (Figure 10A, B). The HSP70-2 mice lack tissue-specific expression of a constitutive isoform of hsp70 involved in spermatogenesis. Here, we observed no differences in depigmentation compared to wild-type C57BL/6 mice, with both sets of animals displaying approximately 15% depigmentation at the vaccination site after 4 weeks (Figure 10B). Taken together, the data indicate a critical and non-redundant role for HSP70i in inducing a depigmentation response.
Figure 9. Inducible Hsp70 knockout mice are resistant to depigmentation after TRP-1 vaccination. (A) Wild-type (WT) and Hsp70-1 knockout (Hsp70-1 KO) mice were gene gun vaccinated five times, every 6 days with DNA encoding either 6 μg of optimized TRP-1 or 3 μg each of optimized TRP-1 and HSP70i and imaged 4 weeks after the final vaccination. (B) Image analysis indicates that wild-type mice depigment significantly more compared with the Hsp70-1 KO mice after either vaccination (vacc), indicating that the expression of HSP70i is required for efficient induction of autoimmunity. Data are presented as mean ± SEM, and were repeated twice with similar results. Student’s unpaired 1-tailed t-test. (*P < 0.05; ***P < 0.001; n = 10 per group).
Figure 10. Alternative HSP70 isoform Hsp70-2 is irrelevant for inducing autoimmune responses. (A) Wild-type (WT) and constitutive Hsp70 knockout (Hsp70-2 KO) mice were gene gun vaccinated five times every six days with 6 µg of either TRP-2 or empty vector (EV; plasmid containing no gene insert) control DNA. (B) No difference in depigmentation was observed between vaccinated (vacc) wild-type and Hsp70-2 KO mice. (C) No differences in cytotoxicity were observed in wild-type or Hsp70-2 KO mice towards TRP-2-pulsed splenocytes. Data are presented as means ± SEM. Student’s unpaired 1-tailed t-test. (NS [no significance]; *P < 0.05; **P < 0.01; n = 10 per group.)
HSP70i increases a cytotoxic response toward melanocyte antigens

To determine whether HSP70i mediates the immune activation of CTLs, wild-type C57BL/6 and Hsp70-1 knockout mice vaccinated with a combination of optimized TRP-1- and HSP70i-encoding plasmids were boosted by two additional vaccinations 3 days apart, and 6 days later were assessed for in vivo cytotoxicity toward the encoded antigen as well as for reactivity toward human TRP-2 (Figure 11A). Gene gun-vaccinated wild-type mice displayed killing of 20.6% more splenocytes pulsed with a peptide derived from optimized TRP-1 compared with Hsp70-1 knockout mice (Figure 11B), indicating that CTL activation had taken place primarily in mice capable of expressing HSP70i. The data also reveal cytotoxicity toward approximately 25% of TRP-2 peptide-pulsed splenocytes, indicating that significant epitope spreading had occurred (Figure 11B). By contrast, there were no differences in cytotoxicity toward peptide-pulsed splenocytes from wild-type and Hsp70-2 knockout mice vaccinated with the TRP-2-encoding plasmid (Figure 10C). This is unlike the Hsp70-1 knockout mice, which displayed less killing toward the antigen. It should be noted that mice vaccinated with optimized TRP-1 display two- to threefold increased cytotoxicity toward the derivative peptide as compared with TRP-2-vaccinated animals and in general achieve greater levels of depigmentation as compared to mice vaccinated with TRP-2 (Figures 9 and 10). Together, these data further demonstrate the unique requirement for inducible HSP70 expression in the skin in precipitating autoimmune vitiligo.
Figure 11. Cytotoxic T lymphocyte (CTL) killing towards melanocyte antigens is reduced in Hsp70-1 knockout mice. (A) For in vivo cytotoxicity assays, mice from Figure 9 were challenged with splenocytes pulsed with immunodominant peptides from TRP-1 or TRP-2 or irrelevant control peptides plus differing concentrations of carboxyfluorescein succimidyl ester. Spleens were harvested 18 h after for analysis of CTL activity by fluorescence-activated cell sorter. Data from individual wild-type (WT) and Hsp70-1 KO mice are shown. (B) 20.6% more cytotoxicity was observed in wild-type mice (55.4%) toward TRP-1-pulsed splenocytes than in Hsp70-1 KO mice (34.8%). Approximately 25% cytotoxicity toward TRP-2-pulsed splenocytes is indicative of epitope spreading. Peptide-pulsed splenocytes from control naïve mice are depicted as (C). Data are presented as mean ± SEM. Student’s unpaired 1-tailed t-test. (*P < 0.05; n = 10 per group).
HSP70i is associated with melanocyte loss and T-cell infiltration in actively depigmenting skin

To further support that depigmentation following HSP70i-induced skin immune reactivity reflects a vitiligo phenotype, we evaluated local differences in immune reactivity in response to HSP70i. We probed skin from wild-type C57BL/6 and Hsp70-1 knockout mice 1 week following booster gene gun vaccinations using antibodies against the pan T-lymphocyte marker CD3 and evaluated the density and location of the stained cells (Figure 12A). The vaccinated skin of wild-type mice contained 58.1% more T cells than Hsp70-1 knockout mice (Figure 12B). Further analysis revealed that the vast majority (>95%) of skin-infiltrating CD3+ cells co-express CD8 in both wild-type and Hsp70-1 knockout mice (Figure 13). Although rare, a few CD3+/CD4+ double-positive cells were detected. Most CD4 expression as found primarily in the dermis could be assigned to a CD3-, non-T-cell population (Figure 13). Consistent with the depigmentation data, immunodetection of the melanocyte antigen TRP-1 revealed a 64% reduction in melanocyte-containing hair follicles in wild-type mice compared with Hsp70-1 knockout mice (Figure 12C, D), supporting the loss of melanocytes in the latter that was not observed in mice lacking Hsp70i. Taken together, the immunohistology data establish the development of vaccine-induced vitiligo in the skin of depigmenting wild-type animals.
Figure 12. **Immunohistology indicates that inducible HSP70 is necessary for T-cell mediated loss of melanocytes.** Treatment of mice engaged in this experiment is described under Figure 9. (A) Image of skin near the dermoepidermal junction from mice 1 week after the final booster gene gun vaccination. CD3+ T cells (arrows) are more abundant near hair follicles (*) of wild-type (WT) than of Hsp70-1 KO mice. Gold particles can also be observed (open arrow). (B) Quantification of T-cell infiltration. (C) Image of skin showing more melanocyte-containing hair follicles in vaccinated Hsp70-1 knockout mice compared to wild-type mice. TRP-1 expressing melanocytes (arrows) are shown within hair follicles (*) in an Hsp70-1 KO mouse. (D) Quantification of melanocyte-containing follicles. Depigmentation coincides with loss of melanocytes and T-cell infiltration only in wild-type mice. Scale bar equals 50 μm. Data are presented as mean ± SEM. Student's unpaired 1-tailed t-test. (*P < 0.05; n = 10 per group).
Figure 13. Immunohistology reveals a greater influx of CD8$^+$ T cells in wild-type versus Hsp70-1 knockout mice. (A) Image of skin from a C57BL6 mouse vaccinated with HSP70i + TRP-1 shown in Figure 9 reveal an abundant number of CD3$^+$ T cells (black arrows) in the dermo-epidermal junction. CD3$^+$CD4$^+$ non-T cells (red arrows) were mainly localized to the dermis. A single CD3$^+$CD4$^+$ T cell is shown in the epidermis (green arrow). (B) A serial section from the same tissue as (A) indicates the vast majority of the T cells are CD8$^+$ (black arrows). (C) Fluorescent staining of skin from a C57BL6 mouse vaccinated with HSP70i + TRP-1 further demonstrate the colocalization of CD3 (red) and CD8 (green) as indicated by yellow (red arrows). CD3$^-$CD4$^+$ non-T cells were mainly confined to the dermis (white arrow). A single CD3$^+$CD4$^+$ T cell is shown (green arrow). (D) Skin from an Hsp70-1 knockout mouse vaccinated with HSP70i + TRP-1 reveal less CD3$^+$CD8$^+$ T cell infiltration (red arrow). CD3$^+$CD4$^+$ non-T cells were also present in the dermis (white arrow). Scale equal 50 µm.
HSP70i is sufficient to support depigmentation in a spontaneous model of autoimmune vitiligo

We next tested whether HSP70i alone is sufficient to establish depigmentation in animals predisposed to developing autoimmune depigmentation, akin to human vitiligo. The T-cell-receptor (TCR) transgenic mouse strain Pmel-1 and C57BL/6 mice were gene gun vaccinated with either HSP70i-encoding plasmid or empty vector control DNA. Six months after the final vaccination, Pmel-1 mice approximately 9 months of age vaccinated with HSP70i-encoding DNA displayed markedly increased depigmentation (3.7-fold ventral; 2.8-fold dorsal) compared with mice vaccinated with control DNA (Figure 14A, B). Pmel-1 mice display subtle depigmentation even after vaccinating with empty vector DNA alone, with 18 and 25% depigmentation ventral and dorsal, respectively (Figure 14A, B). This is similar to depigmentation in animals of that age, which had not been vaccinated (Figure 15). As expected, no depigmentation was observed in wild-type mice gene gun vaccinated with any plasmid (Figure 14A, B). This data set implies that elevated HSP70i expression alone is sufficient to augment the depigmentation process in animals prone to vitiligo development.
Figure 14. HSP70i accelerates depigmentation in vitiligo-prone mice. (A) Wild-type (WT) and Pmel-1 mice were vaccinated three times every 7 days with 4 μg of either HSP70i encoding or empty vector (EV) control DNA. Depicted are ventral and dorsal images of representative vaccinated animals 6 months after the final gene gun vaccination. Depigmentation in wild-type mice occurred within and distal to the site of the gene gun vaccination (B) Upon quantification, Pmel-1 mice receiving HSP70i-encoding DNA displayed significantly more depigmentation compared to control vaccinations and vaccinations in wild-type mice, confirming that focal overexpression of HSP70i is sufficient to induce vitiligo in disease-prone animals. Data are presented as mean ± SEM and were repeated twice with similar results. Student’s unpaired 1-tailed t-test. (***P < 0.001; n = 3 per group).
Figure 15. Gene-gun vaccination alone does not accelerate depigmentation in vitiligo-prone mice. This supplementary image of the Pmel-1 mice from Figure 14 demonstrates similar levels of depigmentation in empty vector vaccinated and unvaccinated Pmel-1 mice. Ventral and dorsal views are shown.
HSP70i provides a durable shift of the dendritic cell population towards an inflammatory subset

We next tested whether HSP70i has lasting effects on monocyte-derivative cell populations. Leukocyte phenotypic profiles were obtained from splenocytes of 9-month-old mice euthanized 6 months after the final vaccination. The mouse splenocytes were stained for lymphocyte markers CD3, CD8, and Thy1.2 and the non-lymphocyte markers CD11b, CD11c, and F4/80. No sustained differences in the abundance of lymphocyte subpopulations were found; yet, we observed marked differences in the abundance of monocyte-derived subpopulations discriminated based on CD11b and CD11c expression levels, shown as R1 through R3 (Figure 16A). The R1 population is characterized by the expression of CD11b<sup>int</sup>/hiCD11c<sup>lo</sup>, typical for macrophages. Intriguingly, we show that the population R1 remained markedly suppressed several months after vaccination with HSP70i (Figure 16B). By contrast, the dendritic cell population R2 (CD11b<sup>int</sup>CD11c<sup>int</sup>), representative of pro-inflammatory dendritic cells, showed an opposite trend (Figure 16B). These data demonstrate that HSP70i can drive the relative abundance of dendritic cell subpopulations with inflammatory versus immunosuppressive potential, associated with autoimmune depigmentation.
Figure 16. HSP70i has prolonged effects on peripheral leukocyte composition.
Splenocytes obtained from Pmel-1 mice 9 months after gene gun vaccination with HSP70i or empty vector DNA was stained to discriminate leukocyte subpopulations, and staining was quantified by flow cytometry. (A) Three distinct populations of cells were observed after gating for CD11b and CD11c cells in the non-lymphocyte population, with high (hi), low (lo), or intermediate (int) levels of expression. Macrophages are observed in the R1 population as CD11b\textsuperscript{int}/hiCD11c\textsuperscript{lo} cells, while dendritic cells are among the R2 and R3 populations as CD11b\textsuperscript{int}CD11c\textsuperscript{int} and CD11b\textsuperscript{hi}CD11c\textsuperscript{hi} cells, respectively. (B) Quantification of CD11b- and CD11c-expressing cells determined differential expression of these markers among leukocytes after vaccination with HSP70i-encoding or empty vector plasmids. The macrophage population (CD11b\textsuperscript{hi}CD11c\textsuperscript{lo}) shown in R1 displays decreased quantitative differences in response to HSP70i. By contrast, the inflammatory dendritic cell population shown in R2 (CD11b\textsuperscript{int}CD11c\textsuperscript{int}) shows an opposite trend. These results demonstrate the differential effects of HSP70i on monocyte derivative leukocyte populations. Student’s unpaired 1-tailed t-test. *P < 0.05, n = 3.
On the basis of the fluorescence-activated cell sorter (FACS) profiles suggesting that HSP70i induced sustained changes in dendritic cell populations, we assessed whether blocking the HSP70i protein could prevent dendritic cell activation. Mouse bone marrow monocytes were magnetically sorted and driven to a dendritic cell phenotype by the addition of granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4 cytokines. After 1 week, HSP70i with or without blocking antibodies was added. To prevent the HSP70i antibody from inducing Fc-receptor–mediated dendritic cell stimulation itself, Fc-receptor-blocking antibodies were included. Lipopolysaccharide (LPS) and control media were used as controls. Twenty-four hours later, the dendritic cells were stained for activation markers CD11c, CD80, CD83, CD86, and MHCII and analyzed by flow cytometry (Figures 17 and 18). Gating on the CD11c+ population, it was observed that the entire population expressed CD80, CD86, and MHCII, with marked increases in expression attributed to the addition of HSP70i or LPS (Figure 18A). By contrast, the addition of antibodies to HSP70i markedly decreased activation (Figure 18A). Staining for CD83 revealed expressing and non-expressing cells (Figure 18A), with increases noted in the CD83+ population after the addition of LPS or HSP70i, and decreased activation with HSP70i antibodies (Figure 18B). Together, these data confirm that the HSP70i protein directly activates dendritic cells, while strongly suggesting that antibodies to HSP70i may be useful in preventing dendritic cell activation in vitiligo.
Figure 17. Antibodies can block HSP70i induced dendritic cell activation \textit{in vitro}. This supplementary image displays the fluorocytometry histograms representing activation marker expression by CD11c+, cultured mouse dendritic cells as summarized in Figure 18. Whereas exposure to HSP70i increases expression of CD80, CD83, CD86, and MHCII, in presence of antibodies to HSP70i, dendritic cell activation is largely prevented.
Figure 18. Antibodies to HSP70i interfere with dendritic cell activation in vitro. Dendritic cells derived from mouse bone marrow monocytes were grown in culture for 7 days followed by the addition of either 1 μg/ml of HSP70i or a combination of HSP70i and Fc-receptor-blocking antibodies. LPS and control media were used as controls. Twenty-four hours later, the cells were stained for the dendritic cell maturation markers CD11c, CD80, CD83, CD86, or MHCII and analyzed by fluorescence-activated cell sorter. Subsequent gating was performed on the CD11c⁺ cells. (A) Mean fluorescent intensity (MFI) indicates higher expression levels of CD80, CD86, and MHCII after the addition of HSP70i as compared to control media, whereas the addition of blocking antibodies was able to reduce expression levels close to baseline. (B) A higher percentage of dendritic cells expressing CD83⁺ were detected after the addition of HSP70i as compared to control media, whereas blocking HSP70i reduced the cell number close to baseline. All data are represented as % stimulation compared to LPS. These results demonstrate the stimulatory effects of HSP70i on dendritic cells, which can be augmented by blocking antibodies.
DISCUSSION

Our current data provide support to the notion that inducible HSP70 is a critical component in mediating autoimmune reactivity to melanocytes in vitiligo-prone animals. Previously, we have shown that overexpression of the heat-shock protein, together with melanocyte-specific target antigen TRP-2 of human origin, could serve to induce progressive depigmentation in mice (Denman et al., 2008). Such depigmentation was originally restricted to the vaccination site but would later diffusely spread to other sites as described (Denman et al., 2008). Melanocytes were not detected in follicles giving rise to depigmented hair, which is similar to the actual loss of melanocytes (rather than reduced melanization by existing melanocytes) observed in human vitiligo skin (Le Poole et al., 1993). The vaccine combining TRP-2 and HSP70i was effectively used to generate an inducible animal model of the disease, useful to study immune effector responses involved in vitiligo. At that time, we did not address the unique or redundant role of HSP70i versus other stress proteins or discuss whether antigens other than TRP-2 can likewise serve to direct immune responses toward melanocytes.

As emphasized by the current data, the role of TRP-2 as a target antigen is not unique; we can replace this antigen by others expressed within the melanosomal compartment to likewise introduce progressive, CTL-mediated responses to melanocytes. This is in concordance with the literature where the application of anti-melanoma vaccines targeting either tyrosinase or TRP-2 was associated with depigmentation of the pelage (Chapman and Wolchok, 2002). Depigmentation in response to DNA vaccines encoding human gp100 was
mentioned previously as well (Chapman and Wolchok, 2002). There is, however, a difference in the type and extent of immune activation elicited by individual antigens. In fact, minimal humoral immunogenicity has been assigned to TRP-1, whereas TRP-2 can drive CTL responses (Avogadri et al., 2010). To elicit robust immune responses to vaccines, the immunogenicity of naturally occurring TRP-1 has been adjusted in its optimized version Tyrp-ee, generating peptides with enhanced binding to MHC and neopeptides revealed by deglycosylation (Guevara-Patino et al., 2006). Indeed, in the current studies, optimized TRP-1 resulted in greater levels of depigmentation, as well as a two- to threefold increase in cytotoxicity as compared to vaccination with TRP-2 in C57BL/6 wild-type mice. Also, following optimized TRP-1 vaccination, responses were observed to TRP-2 derivative peptide SVYDFFWL. This peptide shares homology to the TRP-1 protein spanning the first eight amino acids. As vaccination with native TRP-1 does not induce anti-TRP-2 responses, this phenomenon is most likely because of epitope spreading, with T cells targeting heteroclytic epitopes killing melanocytes, which subsequently results in response to TRP-2 as well. At this time however, we cannot rule out that reactivity to TRP-2 is rather a consequence of cross-reactivity, where optimized TRP-1 has altered its processing in such a way that the homologous TRP-1 derivative peptide directly elicits a response to TRP-2.

The use of Tyrp-ee, eliciting optimal responses to melanocytes, was important for the greatest possible odds of detecting vitiligo in vaccinated animals in the absence of inducible HSP70. Even with this optimized antigen, however, consistent depigmentation was not observed in animals lacking hsp70i, the
inducible form of hsp70. In contrast, depigmentation was uninhibited in mice lacking hsp70-2, a tissue-specific isoform of hsp70 constitutively expressed in testes, even in response to vaccination with suboptimal antigen TRP-2. These findings suggest that HSP70 isoforms expressed outside the skin are less important to vitiligo development.

It is important to note that the progressive loss of melanocytes and T-cell infiltration to the skin are considered hallmarks of vitiligo, and such hallmark events were likewise observed in our depigmenting mice (Badri et al., 1993; Ongenae et al., 2003). Enhanced T-cell infiltration was inversely related to the loss of melanocytes within the wild-type mice, which can be attributed to increases in self-reactive CTLs. Meanwhile, enhanced T-cell infiltration or reduced melanocyte numbers were not observed in animals lacking expression of inducible HSP70. Removing isoform Hsp70-2, however, still resulted in melanocyte death. Taken together, our data demonstrate a critical role reserved for inducible HSP70 in supporting depigmentation.

Regarding potential redundancy of HSP70i, anti-melanoma vaccines are commonly applied in the absence of adjuvant heat-shock proteins. This led us to consider that stress to the skin involved with the vaccine application process may be sufficient to enhance heat-shock protein expression in the skin, serving as a natural adjuvant to enhance autoimmunity. Stress-induced depigmentation is conclusively supported by reports of depigmentation occurring at the site of injection in mice previously vaccinated against melanocyte antigen TRP-2 (Lane et al., 2004). The question addressed here was whether there is a unique role reserved for HSP70i in
autoimmune depigmentation in vitiligo. As other heat-shock proteins including HSP27 and HSP110 have likewise been implicated as adjuvants in anti-melanoma vaccines, a non-redundant role of HSP70i would appear unusual (Srivastava, 2002). Our preliminary data in fact showed that in contrast to HSP60, HSP27 was differentially expressed in vitiligo lesional skin (not shown). This implies that HSP27 may play a role similar to HSP70, where patchy expression of the heat-shock protein was noted in lesional, but not in non-lesional skin (Le Poole and Luiten, 2008). Also, gp96 has been implicated in autoimmune disease (Han et al., 2010), and it has been hypothesized that it is secreted in response to stress or cell death (Li et al., 2002). Investigators have modified gp96 to allow it to be secreted; however, there are no published data available supporting natural secretion of gp96, and this finding remains exclusively reserved for HSP70i (Strbo and Podack, 2008). In contrast to HSP70i, which is generally located in the cytoplasm or nucleus of the cell, gp96 is expressed in the endoplasmic reticulum (Li et al., 2002). The difference in subcellular location and function suggests that in the absence of HSP70i, gp96 cannot simply compensate for a loss of HSP70i. The current data indeed support that compensation does not occur, which is best explained by different circumstances that call for overexpression of either stress protein, as well as its differential role in folding and chaperoning newly synthesized versus resident cellular proteins (Srivastava, 2002).

The current data definitively support a non-redundant role of HSP70i in progressive depigmentation, as we were unable to confer significant depigmentation upon mice that lack expression of the inducible isoform of HSP70.
Although HSP70i was included in the vaccine, focal expression does not restore depigmentation, suggesting that systemic expression of HSP70i is required to drive biologically relevant responses to melanocytes.

We next chose to determine whether HSP70i alone was sufficient to define depigmentation in a model genetically prone to develop vitiligo. In this regard, the recent development of relevant TCR transgenic mouse models now provides an opportunity to study and modulate effector responses in vitiligo. T-cell receptor transgenic animals with the majority of T cells reactive to a TRP-1-derived peptide presented in the context of MHC class II were engaged in experiments to provide a first line of evidence that a newly discovered subset of helper T cells, the Th17 cell, provides a driving force behind immune reactivity to melanocyte differentiation antigens in vitiligo (Muranski et al., 2008). This novel finding has yet to be translated to the human setting, where an infiltrate of CD4 cells may likewise be composed primarily of Th17 cells, as Th17 cells have now been implicated in several autoimmune settings (Hu et al., 2011). The TRP-1 reactive, TCR transgenic model is maintained in cappuccino mice, lacking any expression of the target antigen, as TRP-1-reactive T cells are otherwise clonally deleted (Muranski et al., 2008). Interestingly, clonal deletion does not occur in a setting of MHC class I restricted TCRs. The FH, or ‘FMDGTMSQV specific, high affinity’ mouse expressing TCR transgenic CD8 T cells reactive with mouse tyrosinase is therefore the first reported spontaneous model of vitiligo, with depigmentation initiating by 4 weeks of age (Gregg et al., 2010). This model has been pivotal to identify the central role of IFN-γ in depigmentation (Gregg et al., 2010). Owing to the rapid development of vitiligo,
this model is, however, less amenable to prophylactic measures that may prevent
depigmentation. On the other hand, the Pmel-1 mouse expresses a gp100-reactive
transgenic TCR on the majority of circulating CD8+ T cells, but mice are generally not
maintained to the point where spontaneous depigmentation is observed (Overwijk
et al., 2003). We found that by 9 months of age, minimal yet measurable
depigmentation will occur. Delayed depigmentation can possibly be explained by a
relatively lesser affinity of this TCR for its antigen, allowing T cell to circulate
without causing damage to melanocytes. It is also possible that the cognitive peptide
is less immunogenic or has reduced binding affinity to MHC. Whatever the cause, the
Pmel-1 mouse has provided us with the currently exploited opportunity to study
precipitating factors in vitiligo, as we were able to demonstrate that HSP70i is a
critical player in defining the depigmentation process.

Earlier, it was reported that CD11bintCD11cint dendritic cells induce IL-17-
producing T cells associated with autoimmunity, which fits with our observations
that this dendritic cell population (shown as R2 in Figure 16) remains increased at 6
months after vaccination with wild-type HSP70i (Denning et al., 2007). Further,
Denning et al. (2007) demonstrated that the CD11b+F4/80+CD11clo macrophage
population induced Foxp3+ regulatory T cells (Tregs). Although we recognize the
preliminary nature of our observations, we currently demonstrate that the same
subset of Treg-inducing macrophages is downregulated by HSP70i. Thus, HSP70i
may contribute to vitiligo through the inhibition of Tregs via reduced macrophage
activity and through supporting Th17-mediated autoimmunity by inflammatory
dendritic cells. It appears then that HSP70i may be acting on two fronts, by
activating CTLs via dendritic cell activation and simultaneously downregulating Treg activity.

Taken together, these data suggest that stress signals to the skin are funneled through inducible HSP70 to activate dendritic cells in the immediate environment of the stressful event. Thus, HSP70i may be regarded as a targetable entity, important for the development of new treatment strategies in vitiligo. When considering this opportunity, current knowledge about heat-shock protein-mediated dendritic cell activation can be used for guidance (Basu et al., 2000; Kuppner et al., 2001). Under stress, secreted inducible HSP70 serves as an adjuvant in vitiligo. Indeed, HSP70 has been previously shown to break T-cell tolerance toward self-antigens as mediated by dendritic cells (Millar et al., 2003). Thus, if dendritic cell activation is mediated primarily by HSP70i, such activation may be prevented by preventing the stress protein from binding dendritic cells.

Several stress protein receptors have been identified on the surface of immature dendritic cells. Such receptors include TLR4, TLR2, CD14, CD91, and CD40 (Asea et al., 2000, 2002; Chen et al., 2009). Preventing binding of HSP70i by blocking its receptors through antibodies is not likely to meet with success, as antibody binding will have dendritic cell-activating consequences itself, and may have cytolytic effects on cells other than the pivotal dendritic cell population. Thus, we are currently focusing attention on HSP70i itself and attempting to prevent its binding to dendritic cells. Here, we demonstrate that antibodies to HSP70i prevented complete dendritic cell activation as measured by expression of CD80, CD83, CD86, and MHC class II molecules. It is thus entirely possible that preventing
HSP70i from binding to dendritic cells will provide an effective prophylactic measure in vitiligo. In conclusion, the current data provide evidence of a unique and pivotal role of HSP70i in progressive vitiligo, suggesting that targeting this heat-shock protein can contribute to new treatment of the autoimmune depigmentation process.
CHAPTER V

Mutant HSP70 reverses depigmentation in autoimmune vitiligo

(Mosenson et al., Sci Transl Med, 2013)

INTRODUCTION

Vitiligo is an autoimmune disease that affects about 0.5% of the world population; patients with vitiligo present with progressive skin depigmentation (Das et al., 2001). About 25% more women than men develop the disease (Cedercreutz et al., 2010). Current treatment modalities seldom induce lasting repigmentation. Ablation of autoimmunity is the most commonly prescribed approach, through the use of systemic or topical corticosteroids or topical application of calcineurin inhibitors (tacrolimus, pimecrolimus), often supplemented by ultraviolet (UV) phototherapy (Colucci et al., 2012). Treatment responses are frequently inadequate, and complications from steroid therapy or calcineurin inhibition can occur (Teraki et al., 2012). Melanocyte-protective pseudocatalase treatment does not appear to offer superior efficacy over UV treatment alone (Hossani-Madani and Halder, 2010). Transplantation can remedy local depigmentation only in patients with already stable disease (Fongers et al., 2009). Accordingly, a safe and effective approach that induces immune tolerance to melanocyte differentiation antigens may offer a superior alternative. This prompted
us to identify a molecular entity critically involved in depigmentation and design an intervention method.

Expanding vitiligo lesions are consistently infiltrated with T cells in the areas lining the lesional borders; these infiltrating T cells are composed primarily of CD8+ cytotoxic T cells reactive with melanocyte-specific antigens (Wańkowicz-Kalińska et al., 2003). Because melanocyte-specific antigens are also targeted by T cells in deadly melanoma skin cancers, patients with vitiligo may develop immune responses that affect their odds of developing melanoma. Autoimmune vitiligo patients develop immune responses mediated by T cells expressing high-affinity T cell receptors (TCRs) that may be exploited for melanoma treatment (Oyarbide-Valencia et al., 2006). Indeed, melanoma and other skin cancers appear to occur less frequently in established vitiligo patients (Oyarbide-Valencia et al., 2006). Moreover, increased numbers of CD11c+ dendritic cells (DCs) have been observed in vitiligo perilesional skin, which may lend to an increased vitiligo/antimelanoma response (Kroll et al., 2005).

Inducible heat shock protein 70 (HSP70i) has recently gained attention for its role in precipitating vitiligo (Mosenson et al., 2012). In an initial screening of potential stress protein involvement, HSP70i in nonlesional and lesional skin biopsies provided the most apparent example of differential expression (Le Poole and Luiten, 2008). Depigmentation in mice vaccinated with DNA encoding melanocyte antigens was markedly enhanced in response to the presence of HSP70i-encoding DNA, suggesting the immune-enhancing role of the stress protein (Denman et al., 2008). In knockout mice, the absence of HSP70i greatly affected
their ability to develop progressive depigmentation, which correlated with reduced activation of a CD8+ T cell-mediated response to melanocytes (Mosenson et al., 2012). In addition, the antigen-presenting cell (APC) profile is driven toward an inflammatory repertoire in vitiligo-prone mice after vaccination with $HSP70i$ (Mosenson et al., 2012).

$HSP70i$ can be secreted by live cells of neuronal ancestry (Asea, 2007). Soluble $HSP70i$ can activate DCs, leading to more efficient uptake, processing, and presentation of antigens (Srivastava, 2002). Thus, the interaction between extracellular, soluble $HSP70i$ and recipient DCs will need to be compromised to intervene with depigmentation. A strategy involving targeting of the DC surface is limited by the existence of multiple different receptors for $HSP70i$ on DCs (Asea et al., 2002; Basu et al., 2001; Becker et al., 2002; Wang et al., 2005), and functionally blocking all receptors will have consequences well beyond preventing $HSP70i$ activity. Blocking $HSP70i$ itself at a site otherwise involved in binding DCs may be more successful. Here, we identified an $HSP70i$ variant that prevents the vitiligo-associated inflammatory DC phenotype and averts depigmentation in mice. We conclude that mutant $HSP70i$ is not only an inactive variant but also binds DCs and alters their function to interfere with subsequent T cell activation. The implications of this study are that $HSP70i_{Q435A}$ DNA delivery may be used for the treatment of vitiligo patients.
RESULTS

Vitiligo patients present with inflammatory DCs

DC subsets can be identified on the basis of surface expression of CD11b and CD11c markers and by the cytokines they generate. Inflammatory DCs (CD11b⁺CD11c⁺) produce interleukin-17 (IL-17) and are associated with inflammation, whereas an anti-inflammatory CD11b⁺CD11c⁻ population will generate cytokines including IL-10 and transforming growth factor–β to support regulatory T cell differentiation (Pulendran et al., 2008; Denning et al., 2007). Conventional CD11b⁻CD11c⁺ DCs are particularly supportive of T helper 1 responses and secrete IL12p70 (Pulendran et al., 2008; Denning et al., 2007).

Differential expression of HSP70 in vitiligo skin is accompanied by DC infiltrates (Kroll et al., 2005; Le Poole and Luiten, 2008). Exposure of mice to HSP70i results in increased abundance of inflammatory (CD11b⁺CD11c⁺) DCs (Mosenson et al., 2012). To establish whether the same elevated inflammatory DC content is also found in vitiligo patients (see table 1 for patient information), we stained peripheral blood mononuclear cells (PBMCs) from vitiligo and non-vitiligo controls for CD11b and CD11c expression (after gating out T and B cells). A marked increase in CD11b⁺CD11c⁺ cells (R2) was observed among PBMCs from the vitiligo patients (Fig. 19, A and B; P = 0.04). No differences were found between the two groups among the percent of cells staining for CD11b⁺ or CD11c⁺ alone. To assess whether this inflammatory DC subset is present in skin, we stained biopsies from non-vitiligo controls and nonlesional and perilesional tissue from vitiligo patients for CD11b and CD11c expression to identify different subsets of APCs. A significant increase was
observed in inflammatory CD11b^+CD11c^+ cells in perilesional skin versus nonlesional \((P = 0.0495)\) and non-vitiligo skin \((P = 0.0495)\) (Fig. 19, C to F). The inflammatory DC population was observed mainly in the dermis and toward the dermo-epidermal junction (Fig. 19, C to E). These data reveal a markedly increased abundance of inflammatory CD11b^+CD11c^+ DCs in human vitiligo.
Table 1. Vitiligo patient information. A table showing relevant patient information including treatment is shown. The tissue samples used for data acquisition by fluorocytometry and immunohistochemistry shown in Fig. 19 are indicated.

<table>
<thead>
<tr>
<th>Samples collected</th>
<th>Disease duration</th>
<th>Family history</th>
<th>Current treatment</th>
<th>Gender</th>
<th>Skin involvement</th>
<th>Autoimmune disease</th>
<th>Disease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin (elbow)</td>
<td>2 y</td>
<td>-</td>
<td>Polypodium leucomoros, laser</td>
<td>M</td>
<td>5%</td>
<td>-</td>
<td>Active</td>
</tr>
<tr>
<td>Skin (hip)</td>
<td>15 y</td>
<td>-</td>
<td>Laser, nutrition</td>
<td>F</td>
<td>60%</td>
<td>Hypothyroid</td>
<td>Active</td>
</tr>
<tr>
<td>Skin (arm)</td>
<td>9 y</td>
<td>-</td>
<td></td>
<td>F</td>
<td>15%</td>
<td>Hypothyroid</td>
<td>Active</td>
</tr>
<tr>
<td>Blood</td>
<td>8 y</td>
<td>-</td>
<td></td>
<td>F</td>
<td>2%</td>
<td>-</td>
<td>Active</td>
</tr>
<tr>
<td>Blood</td>
<td>21 y</td>
<td>+</td>
<td>Tacrolimus, pimecrolimus</td>
<td>F</td>
<td>2%</td>
<td>Hypothyroid</td>
<td>Active</td>
</tr>
<tr>
<td>Blood</td>
<td>14 y</td>
<td>-</td>
<td></td>
<td>F</td>
<td>20%</td>
<td>Hypothyroid</td>
<td>Active</td>
</tr>
</tbody>
</table>
Figure 19. APCs in progressive human vitiligo reflect the phenotype observed in response to HSP70i. (A) PBMCs from non-vitiligo (N.V.) control (left flow plot) and vitiligo (right flow plot) patients were gated for expression of CD11b and CD11c after excluding T and B cells. (B) Quantification of the percentage of cells among R1 to R3 indicates an increase in R2 (CD11b+CD11c+) cells among the vitiligo patient samples. (C to E) Non-vitiligo (N.V.) patient (C) and vitiligo patient (D) nonlesional (N.L) and (E) perilesional (P.L) skin were stained for expression of APC markers CD11b (red staining and arrows) and CD11c (blue staining and arrows) with colocalization observed in purple (purple staining and arrows). (F) Quantification of double positive–stained cells indicates a significant increase in CD11b+CD11c+ cells within perilesional skin. Scale bars, 25 μm. Data are presented as the mean ± SEM, n = 3. (A to B) analyzed by Student’s unpaired two-tailed t-test and (C to F) analyzed by Wilcoxon’s rank sum test. Degrees of freedom were calculated using Satterthwaite’s formula. *P < 0.05, **P < 0.01.
Autoimmune stimulatory activity resides in the C terminus of HSP70i

HSP70i is necessary for autoimmune vitiligo in mice (Mosenson et al., 2012). Therefore, we hypothesized that manipulating HSP70i could locally alter the APC profile and affect the resulting immune response. Thus, it was important to identify the DC-activating region in HSP70i. We cloned overlapping sequence fragments of HSP70i encoding either the N-terminal (amino acids 1 to 377) or C-terminal (amino acids 320 to 641) regions. Protein expression was confirmed by Western blot with HSP70i-reactive antibodies (Fig. 20). C57BL/6 mice were then vaccinated with DNA plasmids encoding human melanocyte antigen TRP-2 (hTRP-2), which is capable of inducing temporary, minor depigmentation, combined with DNA encoding full-length HSP70i, N- or C-terminal HSP70i fragments, or empty vector DNA (Fig. 21A). Significant depigmentation was observed in mice vaccinated with full-length HSP70i, which accounted for >14% depigmentation compared to ~2% in the empty vector–vaccinated mice (P = 0.0088; Fig. 21A). The HSP70i C terminus alone achieved markedly greater levels of depigmentation (>40%) than the full-length sequence when compared to empty vector–vaccinated mice (P = 0.0139; Fig. 21A) and N terminus–vaccinated mice (P = 0.0275; Fig. 21A). By contrast, the N terminus did not induce depigmentation (<5%, similar to empty vector–vaccinated mice) (Fig. 21A). The overlapping N- and C-terminal regions are depicted in Fig. 21B.

The DnaK peptide QPSVQIQVYQGEREIAHNK (DnaK407–426) was shown to drive DC activation during inflammation in response to infection, inducing DCs to produce tumor necrosis factor–α and IL-12 (Wang et al., 2005). However, the corresponding DC-activating region within human HSP70i remains unidentified to
date. To further define the region within the C terminus of human HSP70i required for inducing depigmentation, we identified the peptide sequence QPGVLIQVYEGER as maximally homologous with the proposed DC-activating region of microbial DnaK (Wang et al., 2005). To assess this peptide’s actual contribution to autoimmune activation, we generated mutant constructs using site-directed mutagenesis (Fig. 21B). Protein expression from mutant sequences was demonstrated by Western blot with monoclonal antibody SPA-810, generated against a 67-mer partially overlapping the peptide of interest, and polyclonal antibody SPA-811 recognizing a C-terminal peptide downstream (Fig. 20).

We next determined the depigmenting effects of mutant HSP70i in vivo. Wild-type C57BL/6 mice were vaccinated with plasmids encoding wild-type or mutant HSP70i and melanosomal antigen hTRP-2, and depigmentation was assessed 5 weeks after the final vaccination (Fig. 21C). Depigmentation was significantly decreased in the presence of variants HSP70iQ435A (P = 0.0055), HSP70iV438K, I440A (P = 0.0178), and HSP70iV442A, Y443V (P = 0.0015) compared to wild-type HSP70i. This further confirms a peptide of interest consisting of amino acids QPGVLIQVYEG as crucial to immune activation [and excludes amino acids ER (amino acids 446 to 447) as being necessary for depigmentation].

Of interest, antibody SPA-810 demonstrated markedly reduced reactivity toward mutants HSP70iV438K, I440A, HSP70iV442A, Y443V (>60% reduced reactivity), but not HSP70iE446V, R447A compared to the wild-type sequence (Fig. 20, A and B). In concordance with the immunizing peptide used to generate SPA-810, recognition of HSP70iQ435A was not affected (Fig. 20, A and B). Together, the data indicate that SPA-
810 recognizes an epitope that overlaps with our DC activation sequence of interest, yet antibody binding is unaffected by the HSP70iQ435A mutation. Because SPA-810 can inhibit DC activation in vitro, these data provide further support for the involvement of its target sequence in DC activation (Mosenson et al., 2012). Because the HSP70iQ435A mutation does not affect binding of SPA-810, this mutation is well suited for further studies. Further, a three-dimensional representation of HSP70i demonstrates that the Q435A mutation is peripherally located, and thus suggests that it is uniquely positioned to interact with DCs independent of its chaperone function (Fig. 21D, arrow). Therefore, a single amino acid difference in the QPGVLIQVYE peptide is sufficient to inactivate the depigmentation accelerating effects of HSP70i and supports the crucial involvement of the moiety in inducing autoimmune depigmentation.

Mice vaccinated with HSP70i develop a humoral response to the protein (Denman et al., 2008). The exact sequence with HSP70i bound by these circulating antibodies may affect the ability of HSP70i to activate DCs. To determine whether the N or C terminus of HSP70i was the target of humoral responses in mice, we prepared protein homogenates from HSP70i-transfected COS7 cells and electrophoresed them on an SDS-polyacrylamide gel. In serum from the empty vector– and HSP70i1-377–vaccinated mice, antibodies toward HSP70i were not detected, whereas mice vaccinated with either full-length HSP70i or the C terminal (HSP70i320–642) developed antibodies toward the C terminal of HSP70i (Fig. 22A). Further Western blotting revealed that humoral responses were directed toward residues 494 to 641, rather than residues 262 to 551, recognizing C-terminal amino
acids beyond amino acid 551 (Fig. 22B). The data are congruent with the proposed location of the DC-activating region within HSP70i proposed above and explain why endogenous antibodies to HSP70i may not affect DC binding and activation mediated by HSP70i. The C terminus is thus required for inducing depigmentation and is more immunogenic than the full-length HSP70i.
Figure 20. SPA-810 antibody recognizes the DC activating region of HSP70i. (A) A Western blot showing relative recognition by HSP70i reactive antibody. HSP70i bands reacted with SPA-810 and SPA-811 are visible at 70 KDa, and actin bands are observed at 55 KDa. (B) The mutant protein HSP70iQ435A is recognized with equal efficiency as wild-type HSP70i (WT) whereas monoclonal antibody SPA-810 shows 60-65% reduced reactivity towards mutant HSP70i plasmids HSP70iV438K, I440A and HSP70iV442A, Y443V as calculated by comparing band intensities to WT observed using antibody SPA-811 which binds downstream of the DC activating region. Thus the SPA-810 binding epitope includes the sequence PGVLIQVYEG. Data are presented as the mean ± SEM, and were repeated twice with similar results (n = 2). Student’s unpaired one-tailed t-test. * P < 0.05, *** P < 0.001).
Figure 21, Part I. A peptide within the C terminus of HSP70i is required for inducing depigmentation. Continued on page 93.
Figure 21, Part II. A peptide within the C terminus of HSP70i is required for inducing depigmentation. Continued on page 93.
Figure 21. A peptide within the C terminus of HSP70i is required for inducing depigmentation. Part I, (A) To identify the region of HSP70i critical for inducing depigmentation, mice were vaccinated with DNA plasmids encoding wild-type (WT) N terminus, C terminus, or full-length HSP70i. Mice vaccinated with HSP70i N terminus (residues 1 to 377) expressed depigmentation similar to the empty vector control-treated animals. Mice vaccinated with the HSP70i C terminus displayed far greater levels of depigmentation than those exposed to full-length HSP70i plus hTRP-2 vaccination (n = 5). Representative images of C57BL/6 mice imaged 4 weeks after vaccination with DNA-encoding melanosomal antigen hTRP-2 and either full-length HSP70i, N-terminal (N-term) or C-terminal (C-term) HSP70i fragments, or empty vector (EV) are shown. Mice were vaccinated five times, every 7 days, with 5.6 μg of total DNA. (B) Schematic of the N-terminal ATP-binding domain (ABD) and C-terminal substrate-binding domain (SBD) of HSP70i and fragments (HSP70i1–377 and HSP70i320–641) and mutations introduced within the putative DC-activating region. Part II, (C) No depigmentation was observed in mice receiving vaccinations with hTRP-2 and several mutant versions of HSP70i compared to hTRP-2 and WT HSP70i, supporting the crucial involvement of the 11-mer with the exceptions of amino acids 446 and 447. Representative images of mice 5 weeks after vaccination (n = 10) are shown. Mice were vaccinated four times, every 7 days, with 4 μg of total DNA. (D) Three-dimensional representation showing the peripheral location of the mutant Q435A (magenta) residue (red arrow) ideally located at the interface of the ATP- and substrate-binding domains. Data are presented as the mean ± SEM. Numeric data were analyzed for statistical significance using two-tailed Wilcoxon’s rank sum test, and degrees of freedom were calculated using Satterthwaite’s formula. *P < 0.05, **P < 0.01.
Figure 22. The C-terminus induces a humoral response to HSP70i in vaccinated mice. (A) Serum was collected from mice vaccinated with full length or fragmented HSP70i encoding DNA plasmids and used to probe proteins from COS7 cells transfected with either N-terminus (N-term) or C-terminus (C-term) of HSP70i DNA. Bands corresponding to the C-terminal peptide (36 kDa; amino acids 320-641) and full length HSP70i (70 kDa) were revealed only by sera from mice vaccinated with the C-terminus or full length HSP70i. No bands corresponding to the N-terminal peptide (41 kDa; amino acids 1-377) were revealed. (B) Likewise, bands corresponding to the HSP70i C-terminal peptide 494-641 which is downstream of the DC activation region were revealed only by sera from mice vaccinated with the C-terminus or full length HSP70i. (EV: Empty vector). n= 5 mice, with serum pooled for each group.
Mutant HSP70i interferes with DC activation

To determine the role of the QPGVLIQVYEG moiety in DC activation, we vaccinated wild-type C57BL/6 mice and vitiligo-prone TCR transgenic Pmel-1 mice (Overwijk et al., 2003) using plasmids encoding either wild-type HSP70i, HSP70i_{Q435A}, or empty vector DNA. The Q435A mutation was used because of its position potentially affecting DC binding and activation (Fig. 21D). Splenocytes were stained for expression of markers CD11b and CD11c after excluding T and B cells. No additional gating for monocytes was performed. We observed marked differences in the abundance of monocyte-derived subpopulations based on CD11b and CD11c expression levels shown as R1 to R3 (Fig. 23, A and B). The R1 population is characterized by expression of CD11b^{+}CD11c^{-}, typical for regulatory DCs (Denning et al., 2007). Compared to HSP70i-based vaccination, we observed significant skewing toward a greater R1 population after HSP70i_{Q435A} vaccination (P = 0.022; Fig. 23B). There was also a trend toward increasing activation after HSP70i_{Q435A} vaccination compared to empty vector (P = 0.0618; Fig. 23B). By contrast, the DC populations R2 (CD11b^{+}CD11c^{+}), representative of proinflammatory DCs, and R3 (CD11b^{-}CD11c^{+}), representative of classic inflammatory DCs (Denning et al., 2007), showed opposing changes (Fig. 23B; P = 0.028 and P = 0.0029, respectively). Overall, these data indicate that wild-type HSP70i and HSP70i_{Q435A} oppositely drive the relative abundance of immune cell subpopulations associated with autoimmune depigmentation in mice.
Mutant HSP70i inhibits depigmentation in vitiligo-prone mice

Six months after vaccination, Pmel-1 mice exhibited spontaneous depigmentation, yet animals exposed to wild-type HSP70i displayed significantly increased ventral and dorsal depigmentation compared to HSP70iQ435A or empty vector \((P = 0.0495; \text{Fig. 23, C, D and E})\). As expected, in the absence of a targetable antigen, no depigmentation was observed in vaccinated C57BL/6 mice (Fig. 23, C, D and E). Depigmentation was significantly prevented in Pmel-1 mice vaccinated with HSP70iQ435A DNA compared to mice vaccinated with empty vector \((P = 0.0495; \text{Fig. 23, C, D, and E})\). Thus, a single amino acid variation in the putative DC binding region of HSP70i is sufficient to actively interfere with immune activation and subsequent depigmentation in vivo.

On the basis of our observations that HSP70iQ435A can prevent depigmentation in mice prone to delayed and slowly progressing depigmentation, we next assessed whether HSP70iQ435A can be therapeutic in the context of existing vitiligo. We treated the early and rapidly depigmenting mouse strain h3TA2, which expresses T cells bearing a human tyrosinase-reactive TCR transgene and HLA-A2.1 (Mehrotra et al., 2012). Depigmentation initiates by about 4 weeks of age, accompanied by a marked loss of melanocytes in 5-week-old animals (Fig. 24). Treatment with HSP70iQ435A or empty vector DNA was initiated at 5 weeks of age. Images of representative mice comparing pre- and postvaccination are shown in Fig. 24. Eight weeks later, the pelage of h3TA2 mice unvaccinated or vaccinated with empty vector DNA was 86% depigmented (Fig. 23, F and G). By contrast, an astonishing 76% of depigmentation in the pelage of mice was restored with mutant
HSP70\textsubscript{iQ435A} and the pelage returned almost fully pigmented ($P = 0.0143$; Fig. 23, F and G, and Fig. 24). Thus, HSP70\textsubscript{iQ435A} can reverse the depigmenting phenotype, critical for treatment of active disease.
Figure 23, Part I. WT HSP70i accelerates and mutant HSP70i prevents depigmentation in vitiligo-prone mice. Continued on page 100.
Figure 23, Part II. WT HSP70i accelerates and mutant HSP70i prevents depigmentation in vitiligo-prone mice. Continued on page 100.
Figure 23. WT HSP70i accelerates and mutant HSP70i prevents depigmentation in vitiligo-prone mice. Part I, (A) Tolerogenic DCs are induced in response to HSP70i\textsubscript{Q435A}. (B) Representative flow cytometry plots and cumulative graphs of mice vaccinated three times in total, every 7 days, with 4 μg of WT, HSP70i\textsubscript{Q435A} (Q435A), or empty vector (EV) control DNA. Splenocytes from Pmel-1 mice (n = 3) 6 months after vaccination were analyzed by flow cytometry. Gating for CD11b and CD11c expression among nonlymphocytes, we observed three distinct populations that vary in abundance in response to the treatments. Differential expression of CD11b and CD11c was found after vaccination with WT or HSP70i\textsubscript{Q435A} plasmids. In contrast to WT HSP70i, the anti-inflammatory population (R1) displays increased, and the inflammatory population (R2) displays decreased, abundance in response to mutant HSP70i-encoding DNA. (C) Ventral and (D) dorsal images were obtained 6 months after treatment. Pmel-1 mice depigmented within and distal to the site of vaccination. (E) Cumulative graphs of mice in (C and D). Pmel-1 mice receiving mutant HSP70i displayed significantly less depigmentation compared to WT HSP70i or empty vector–exposed C57BL/6 mice, confirming inhibition by mutant HSP70i. Part II, (F) To test the therapeutic effects of HSP70i\textsubscript{Q435A} in rapidly depigmenting vitiligo-prone mice, tyrosinase-responsive TCR transgenic h3TA2 mice were vaccinated five times, every 6 days, with 5.6 μg of either empty vector– or HSP70i\textsubscript{Q435A}–encoding DNA plasmid. Mice were imaged 4 weeks after the final vaccination at just over 9 weeks of age. (G) Cumulative data from (F) demonstrating that empty vector–vaccinated mice had significantly more depigmentation than the HSP70i\textsubscript{Q435A}–vaccinated mice (n = 4). Data are presented as the mean ± SEM, and were repeated twice with similar results. Numeric data were analyzed for statistical significance using Wilcoxon’s rank sum test for depigmentation, with degrees of freedom calculated using Satterthwaite’s formula, and Student’s two-tailed t-tests allowing for unequal variance for fluorocytometry. *P < 0.05, **P < 0.01.
Figure 24. Skin melanocytes are lost prior to treatment in h3TA2 mice. (A) Dorsal images of 4-week-old C57BL/6 and h3TA2. Depigmentation is already observed in the ears. (B) Skin biopsies from C57BL/6 reveal melanocytes (red staining) in the hair follicles (arrows) not present within the h3TA2 mice. These images reveal that melanocyte death (and subsequent depigmentation) was already present in the skin of h3TA2 mice prior to treatment. (C) Representative pre and post-vaccination images of h3TA2 mice with DNA encoding empty (EV) and HSP70iQ435A. Bar indicates 50 µm.
Mutant HSP70i prevents accumulation of antigen-specific T cells in the skin

T cell activity mediates the loss of melanocytes in patients with vitiligo (Le Poole et al., 1996). To analyze the local immune-modulating effects of wild-type HSP70i and HSP70iQ435A DNA exposure, we probed the skin of the Pmel-1, C57BL/6, and h3TA2 mice for T cell infiltration. Skin from Pmel-1 mice contained significantly more CD3+ T cells in response to wild-type HSP70i compared to either empty vector- or HSP70iQ435A-vaccinated mice, and skin-infiltrating T cells were primarily located in the dermis and hair follicles ($P = 0.0495$; Fig. 25, A and E). By contrast, a trend toward decreased T cell infiltration was observed in response to HSP70iQ435A vaccination (Fig. 25, B and E). Skin from the h3TA2 mice contained significantly fewer CD3+ T cells in response to HSP70iQ435A ($P = 0.03$; Fig. 25, C, D, and F). We next examined cytotoxic T lymphocyte infiltrates held responsible for melanocyte killing by staining for CD8. HSP70iQ435A vaccination significantly decreased this population in Pmel-1 mice compared to HSP70i ($P = 0.0495$; Fig. 25, G, H, and K) and showed a decreasing trend compared to empty vector-exposed mice. The number of melanocyte-reactive antigen-specific T cells was also significantly reduced in HSP70iQ435A-treated h3TA2 skin ($P = 0.03$; Fig. 25, I, J, and L) corresponding to pigment retention. Together, these data reveal a direct effect between skin exposure to HSP70iQ435A and decreased T cell infiltration resulting in pigment retention.

To further demonstrate a role for T cells in mediating depigmentation, we vaccinated CD4 and CD8 knockout mice using DNA plasmids encoding either wild-type HSP70i, HSP70iQ435A, or empty vector in combination with antigenic mouse Tyrp1 ee (optimized TRP-1) (Guevara-Patino et al., 2006). Wild-type HSP70i
significantly supported depigmentation only in C57BL/6 control mice compared to empty vector ($P = 0.0088$; Fig. 26) and $HSP70iQ_{435A}$ ($P = 0.0493$; Fig. 26). By contrast, depigmentation was not observed in the CD4 or CD8 knockout mice, demonstrating the requirement for both helper and cytotoxic T cells in depigmentation (Fig. 26).
Figure 25. Decreased T cell infiltration is observed after HSP70i\textsubscript{Q435A} vaccination. (A) Pmel-1 mice vaccinated with empty vector (EV) DNA showed abundant skin infiltration by CD3\textsuperscript{+} T cells (arrows) localized mostly to hair follicles (asterisks). (B) CD3\textsuperscript{+} T cell infiltrates seen in (A) were decreased after HSP70i\textsubscript{Q435A} (Q435A) administration. (C and D) T cell infiltrates in skin of h3TA2 mice in response to empty vector and (D) HSP70i\textsubscript{Q435A}. (E) Cumulative data showing changes in skin-infiltrating T cells in treated mice. (F) Quantification of T cell infiltrates in h3TA2 mice. (G) Most of the skin-infiltrating T cells were cytotoxic. (H) In the Pmel-1 mice, CD8\textsuperscript{+} infiltrates in response to empty vector decreased after HSP70i\textsubscript{Q435A} exposure. (I) Reduced number of melanocyte-reactive T cells was observed in response to HSP70i\textsubscript{Q435A}. T cells were quantified on the basis of expression of the tyrosinase transgene–reactive TCR V\textbeta\textsubscript{12} subunit. (J) A decrease in V\textbeta\textsubscript{12}+ cells was observed after vaccination with HSP70i\textsubscript{Q435A}. (K) Quantification of cytotoxic T cell infiltrates in C57BL/6 and Pmel-1 mice, n =3 mice per group. (L) Quantification of melanocyte-reactive T cells in h3TA2 mice, n =5 mice per group. Data are presented as the mean ± SEM. Numeric data were analyzed for statistical significance using Wilcoxon’s rank sum two-tailed test, with degrees of freedom calculated using Satterthwaite’s formula. Scale bars, 50 μm. *P < 0.05.
Figure 26. T cells are required for autoimmune depigmentation. (A) Representative images of C57BL/6, CD4 knockout, and CD8 knockout mice four weeks after vaccination with empty vector (EV), wild-type (WT), or HSP70i₉₄₃₅A (Q435A) plus mouse Tyrp1ee (optimized TRP-1) DNA. Mice were vaccinated five times, every six days with 5.6 μg total DNA. C57BL/6 mice displayed significantly more depigmentation with WT DNA compared to EV and Q435A. No depigmentation was observed in the CD4 or CD8 knockout mice with any of the vaccinations. (B) Cumulative graphs of mice in (A). n=5; Data are presented as the mean ± SEM. Numeric data were analyzed for statistical significance using Wilcoxon’s rank sum two-tailed test, and degrees of freedom were calculated using Satterthwaite’s formula. * P < 0.05.
**Mutant HSP70i interferes with T cell activation**

The relative expression of the transcription factors Eomes and T-bet has been shown in T cells to define a quiescent versus effector status (Rao et al., 2010). T cells from the HSP70iQ435A-treated Pmel-1 mice were analyzed for these transcription factors. Although T-bet expression remained similar among the mouse groups, Eomes expression was lower among CD8+ T cells from HSP70i-vaccinated Pmel-1 mice (versus empty vector–vaccinated mice, $P = 0.039$; Fig. 27A) and showed a decreasing trend compared to HSP70iQ435A vaccination ($P = 0.05$; Fig. 27A), reflecting a more apparent effector phenotype. By contrast, treatment with HSP70i induces an effector phenotype resulting in a significant depression of the Eomes to T-bet ratio despite minimal changes in T-bet alone ($P = 0.035$ compared to HSP70iQ435A, and $P = 0.02$ compared to empty vector; Fig. 27, B and C).

In the previous experiment, we demonstrated that the Eomes/T-bet ratio in mice reflects an effector T cell status after vaccination with HSP70i. Because the immune profiles in vitiligo patients appear to reflect HSP70i-induced changes, we next examined T cell profiles in vitiligo patient tissues. T cells from non-vitiligo and vitiligo patients were stained for Eomes and T-bet, and analyzed by flow cytometry (Fig. 27, D and E). We observed a decreasing trend in the Eomes/T-bet ratio, again indicating an effector response in the periphery of vitiligo patients ($P = 0.06$; Fig. 27F). Similarly, perilesional skin from vitiligo patients displays decreased Eomes expression by CD8+ T cells ($P = 0.0495$; Fig. 27, G and H) as well as increased T-bet expression ($P = 0.0495$; Fig. 27, I and J) compared to nonlesional and non-vitiligo control skin. As with the HSP70i-vaccinated mice, reduced Eomes/T-bet ratios were
observed in the skin of vitiligo patients ($P = 0.0495$; Fig. 27K). We also determined that human vitiligo perilesional skin contains more cells expressing the homing receptor CXCR3 cells than nonlesional skin ($P = 0.02$; Fig. 28), again correlating with the role assigned to this gene product in a mouse model of vitiligo (Gregg et al., 2010).
Figure 27, Part I. HSP70i-treated mice display a similar effector T cell phenotype as vitiligo patient tissue. Continued on page 111.
Figure 27, Part II. HSP70i-treated mice display a similar effector T cell phenotype as vitiligo patient tissue. Continued on page 111.
Figure 27. HSP70i-treated mice display a similar effector T cell phenotype as vitiligo patient tissue. Part I, (A) A decreased percentage of splenocytes from the WT HSP70i-exposed Pmel-1 mice (Fig. 23) expresses the T cell transcription factor Eomes, supporting an enhanced effector T cell phenotype. (B and C) Combined with T-bet expression (C), the resulting Eomes/T-bet ratio further indicates increased effector responses in mice exposed to HSP70i (n = 3). Part II, (D and E) Representative histograms of human PBMCs from non-vitiligo (N.V.) control and vitiligo patients similarly analyzed for Eomes and T-bet expression. (F) The Eomes/T-bet ratio indicates an effector response in vitiligo peripheral blood compared to non-vitiligo control patient samples. (G and H) Nonlesional (N.L.) (G) and perilesional (P.L.) (H) vitiligo skin was stained for T-bet (red staining and arrows) and CD8 (blue staining and arrows) expression, with the cytotoxic T cells shown in purple (purple staining and arrows). A significantly larger number of CD8⁺/T-bet⁺-expressing cells were observed in the dermis of perilesional skin. (I and J) Nonlesional (I) and perilesional (J) skin stained for Eomes (red staining and arrows) and CD8 (blue staining and arrows). Eomes-expressing CD8 cells were significantly increased in non-vitiligo control and nonlesional skin from vitiligo patients. (K) The ratio of Eomes/T-bet-expressing CD8 cells indicates an effector response in perilesional vitiligo skin, similar to that seen in the periphery. n = 3. Data are presented as the mean ± SEM. Numeric data were analyzed for statistical significance using Wilcoxon’s rank sum two-tailed test for immunohistochemistry, with degrees of freedom calculated using Satterthwaite’s formula. Numeric data were analyzed for statistical significance using Student’s two-tailed t-tests allowing for unequal variance for fluorocytometry. *P < 0.05, **P < 0.01.
Figure 28. The T cell homing antigen CXCR3 is overexpressed in actively depigmenting vitiligo skin. Image of nonlesional (A) and perilesional (B) tissue displaying CXCR3+ cells (arrows) in the dermis. (C) Image analysis indicates that perilesional skin contains more CXCR3 expressing cells than nonlesional and control skin. n=3; Data are presented as the mean ± SEM. Numeric data were analyzed for statistical significance using Wilcoxon's rank sum two-tailed test, with degrees of freedom calculated using Satterthwaite’s formula. * P < 0.05.
Wild-type, but not mutant, HSP70i activates human DCs in vitro

Next, we compared the DC-stimulatory effects of wild-type and mutant HSP70i in vitro. Human PBMCs were magnetically sorted and driven toward immature DCs by addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 cytokines (O’Neill and Bhardwaj, 2001). After 1 week, His-labeled wild-type HSP70i or HSP70iQ435A full-length protein, or commercially obtained wild-type HSP70i was added. Twenty-four hours later, the DCs were analyzed for CD11c, CD80, CD83, CD86, and HLA-ABC expression by flow cytometry (Fig. 29, A to E). Among the entire CD11c⁺ population, increases in expression of all DC activation markers were observed in presence of HSP70i or lipopolysaccharide (LPS) (Fig. 29, A to E). By contrast, addition of HSP70iQ435A decreased expression of the activation markers (Fig. 29, A to E).

The previous findings show that HSP70iQ435A protein directly affects DC activity. To determine whether HSP70iQ435A can bind DCs despite the mutation, we used ImageStream analysis, allowing us to quantify expression of a marker by fluorocytometry and localize its expression by confocal analysis within the same set of cells. Antibodies toward HSP70i (SPA-820) and the His tag were used to determine relative binding of wild-type and HSP70iQ435A to DCs (Fig. 29E). Indeed, we detected labeling by both antibodies in the wild-type and also HSP70iQ435A-treated samples, whereas media alone samples contained background levels of fluorescence (Fig. 29, E and F). Together, these data confirm that HSP70iQ435A binds to human DCs and actively interferes with activation marker expression.
**Figure 29. Mutant HSP70i suppresses human DC activation.** (A) Representative histogram displaying HLA-ABC expression from human PBMCs differentiated into immature DCs via GM-CSF and IL-4 and exposed to His-tagged WT or HSP70iQ435A (Q435A) protein (1 μg/ml). Commercially purchased HSP70i [WT(C)], LPS (1 μg/ml), and complete media served as controls. CD11c+ cells were analyzed for expression of CD80, CD83, CD86, or HLA-ABC and analyzed by flow cytometry. (B to E) Changes in mean fluorescence intensity (MFI) between treatments indicating increased expression of activation markers after addition of His-tagged isolated WT HSP70i and commercially purchased HSP70i [WT(C)] and reduced expression of all activation markers after addition of HSP70iQ435A compared to control media. (F) ImageStream images of DCs after the addition of His-tagged WT or HSP70iQ435A (Q435A), or media alone. Cells were stained with antibodies (Ab) toward HSP70i (SPA-820) or the His tag. Single and overlaid channel images are displayed. (G) Similar MFIs using the anti-His antibody show that DCs bind both mutant and WT HSP70i.
HSP70i activates transcriptional and homing factors in human skin

To translate responses to HSP70i\textsubscript{Q435A} toward treatment of human disease, we exposed human skin samples ex vivo and followed the profiles of collected immune cells by fluorocytometry. Skin explants were treated with or without wild-type or mutant HSP70i protein added to the media. Skin T cells were analyzed by flow cytometry 6 days later. Eomes expression was up-regulated in response to HSP70i\textsubscript{Q435A} ($P = 0.02$; Fig. 30A) and wild-type HSP70i ($P = 0.0027$; Fig. 30A). By contrast, a trend toward decreased T-bet expression was visible only in response to HSP70i\textsubscript{Q435A} (Fig. 30B). As we have shown in treated mice (Fig. 27C), HSP70i\textsubscript{Q435A} resulted in a shift of the Eomes/T-bet ratio from an effector to a quiescent phenotype ($P = 0.0154$ compared to HSP70i, and $P = 0.02$ compared to media only; Fig. 30C). In addition, the T cell skin-homing marker cutaneous lymphocyte-associated antigen (CLA) was up-regulated in response to wild-type HSP70i ($P = 0.0147$ compared to HSP70i\textsubscript{Q435A}, and $P = 0.0327$ compared to media only; Fig. 30D), congruent to observations in vitiligo donors (Wijngaard et al., 2000).

We also examined whether T cell activation can be abrogated by HSP70i\textsubscript{Q435A} in human peripheral blood samples. To measure antigen-specific T cell responses, we took advantage of preexisting cytomegalovirus (CMV)–reactive T cells from CMV\textsuperscript{+} HLA-A2\textsuperscript{+} donors. PBMCs were pulsed with CMVpp65 peptide and cocultured with wild-type or HSP70i\textsubscript{Q435A}. After 48 hours, the cells were analyzed by flow cytometry. As in the skin explant model (Fig. 30C), Eomes expression was up-regulated in response to HSP70i\textsubscript{Q435A} ($P = 0.0041$; Fig. 31A) and wild-type HSP70i ($P = 0.0244$; Fig. 31A). By contrast, T-bet was significantly decreased in response to
HSP70i_Q435A (P = 0.0095; Fig. 31B) and increased in response to HSP70i (P = 0.0299 compared to media only, and P = 0.027 compared to HSP70i_Q435A; Fig. 31B). HSP70i_Q435A resulted in a notable shift of the Eomes/T-bet ratio from an effector to a quiescent status (P = 0.0262 compared to HSP70i, and P = 0.0216 compared to media only; Fig. 31C). Likewise, expression of the homing receptor CLA was significantly up-regulated in response to wild-type HSP70i (P = 0.0147 compared to HSP70i_Q435A, and P = 0.0327 compared to media only; Fig. 31D).

We also demonstrated that human skin cells express both wild-type and mutant HSP70i proteins after biolistic transfection (Fig. 32), indicating that DNA vaccination can affect human skin. This may be an important step in bringing HSP70i_Q435A to the clinic. Together, these data reflect that the immune response in human tissues can be differentially driven by HSP70i, which may provide a crucial step in treating vitiligo.
Figure 30. WT and mutant HSP70i differentially activate human skin T cells. Skin explants were maintained submerged in control media supplemented with or without 4 μg of WT or HSP70i_{Q435A} (Q435A) protein for 6 days, and T cell profiles were analyzed after gating for CD3 expression. (A) Eomes expression was significantly up-regulated in T cells from WT and mutant HSP70i-treated skin T cells. (B) T-bet expression is reduced among T cells in response to HSP70i_{Q435A}. (C) Together, the Eomes/T-bet ratios reveal an effector versus memory phenotype in response to WT and mutant HSP70i, respectively. (D) The T cell–homing receptor CLA is highly expressed among T cells in response to WT HSP70i. n = 2. Data are presented as the mean ± SEM. Numeric data were analyzed for statistical significance using Student’s two-tailed t-tests allowing for unequal variance. *P < 0.05, **P < 0.01.
Figure 31. Wild-type and mutant HSP70i differentially activate antigen-challenged human peripheral blood cells. PBMCs from healthy CMV IgG+ donors were cultured in media only, or supplemented with 4 μg of wild-type (WT) or mutant HSP70i (Q435A), with or without 1 μg of CMVpp65 peptide. After 48 hours, PBMCs were gated for CD8 and analyzed for T cell marker expression. (A) Eomes expression was significantly increased among wild-type HSP70i and HSP70iQ435A treated PBMCs. (B) T-bet expression is significantly increased in response to HSP70i and lowered in response to HSP70iQ435A. (C) Together, the Eomes/T-bet ratios indicates an enhanced memory response in wild-type versus mutant HSP70i treated PBMCs respectively. (D) HSP70i enhanced expansion of the T cell homing receptor CLA is increased in response to wild-type HSP70i. n=2; Data are presented as the mean ± SEM. Numeric data were analyzed for statistical significance using Student’s two-tailed t-tests allowing for unequal variance. * P < 0.05, ** P < 0.01.
Figure 32. Transfected human skin expresses HSP70i. Skin biopsies were biolistically DNA-transfected with WT HSP70i (WT), HSP70i_{Q435A} (Q435A), or empty vector and analyzed by flow cytometry 4 days later for expression of HSP70i. Histograms display the relative expression and MFI for each vaccination. Note marked expression of HSP70i as well as its mutant variant among cells collected from adult skin with the respective constructs. $n = 3$. 
DISCUSSION

Differential expression of HSP70i has been observed among nonlesional and lesional human skin samples from vitiligo patients, which prompted us to investigate the phenotype of infiltrating DCs in perilesional vitiligo patient skin. A prevalence of CD11b\(^+\)CD11c\(^+\) DCs as observed here is congruent with our hypothesis that HSP70i secreted under stress will activate local DCs and induce an inflammatory phenotype as previously observed in mice in response to the HSP70i vaccine (Mosenson et al., 2012), and this inflammatory CD11b\(^+\)CD11c\(^+\) DC subset may provide a target for therapy. To better understand the role of HSP70i in autoimmune activation, and identify a region that may be blocked from binding and activating DCs, we generated DNA constructs containing either the N or the C terminus of the molecule, in effect comprising the adenosine triphosphate (ATP)– and substrate-binding domains. Indeed, there is discussion in the field as to the region within stress proteins most important to immune activation. Although protein fusion molecules have been generated that include only the substrate-binding region, others have suggested that the ATP-binding domain is required and sufficient for immune activation (Wang et al., 2002; Zhang et al., 2006). In part, this will be defined by the necessity to include an antigenic moiety that provides direction for the immune response to follow (Javid et al., 2007). Here, the C terminus, including the substrate-binding domain and some residues from the ATP-binding region, had even more pronounced effects than the full-length molecule. Subject to further analysis, it is possible that the compact size or folding of the resulting molecule, optimally exposing its immune-activating region, positively
affects its DC binding ability.

The DC-activating peptide identified within HSP70i was derived by aligning the molecule to DnaK, looking for a sequence with high homology (Wang et al., 2005). Among mutant plasmids, some residues proved more critical than others, ultimately supporting the importance of our 11-mer for DC activation. The QPGVLIQVYEG sequence is shared among HSP70 isoforms and is highly conserved among species from protists to mice to humans (Germot and Philippe, 1999; Adams et al., 1993).

Among the HSP70i mutations that affected immune function, we initially selected HSP70iQ435A to further examine using in vivo effects in vitiligo-prone mice. Its position within the periphery of the molecule, strategically positioned to affect DC binding independent of bound substrate, together with the change from a charged, hydrophilic moiety (Q) to a smaller, hydrophobic one (A), suggests a potential role for enhanced aggregation or preferential receptor binding in the observed effects. This is important particularly as this moiety can affect the preferred receptor for wild-type versus mutant HSP70i. It stands to reason that engaging and disengaging DCs involves activation and possibly blocking of cell surface receptors. DC activation has been related to the relative engagement of C-type lectin receptors (CLRs) and Toll-like receptors (TLRs), with autoimmune pathology resulting from simultaneous activation of both and tolerance resulting from CLR activation in the absence of TLR engagement (Geijtenbeek et al., 2004). Knowing that HSP70i can bind CLRs, including LOX1, as well as TLRs, including TLR2 and TLR4 (Delneste et al., 2002), we can infer that the differential effects
observed for mutant and wild-type HSP70i in these studies may be explained at least in part by differential binding to these receptors, resulting in a different DC phenotype and, in turn, in differential T cell activation and recruitment to the skin. This implies that both wild-type and mutant HSP70i bind DCs, which we showed here.

In Pmel-1 mice, T cells do not become spontaneously activated early on, possibly partly due to differences between the human epitope used to generate the model and the mouse epitope targeted in autoimmunity (Overwijk et al., 2003). These animals are thus ideal to study depigmentation after activation of melanocyte-reactive T cells, much as this occurs in humans. In this model, HSP70i is sufficient to induce depigmentation (Mosenson et al., 2010). However, natural depigmentation did not develop in animals vaccinated with \textit{HSP70i_{Q435A}}. Instead, we observed persistent skewing of the DC phenotype toward the inflammatory subset in response to wild-type HSP70i and encountered this subset among PBMCs and in skin of human patients with progressive disease. Conversely, we observed persistent skewing of the DC phenotype toward the tolerogenic subset in animals vaccinated with \textit{HSP70i_{Q435A}}. Also, HSP70i_{Q435A} exposure similarly affected human DC activation in vitro. Our data support the concept that the direct effect of HSP70i_{Q435A} is on DCs. The Q435A mutation may affect the ability of the resulting stress protein to bind and activate DCs based on differential engagement of receptors described for HSP70i (Zitzler et al., 2008).

We postulate that the resulting DC phenotypes support different cytokine profiles, necessary to activate or suppress relevant T and B cell subsets. Indeed
differential Eomes/T-bet ratios as observed are likely to affect the effector phenotype and quiescent potential of resulting cytotoxic T cells (Rao et al., 2010). It has been shown that T-bet is the master regulator of type I effector differentiation, whereas low T-bet expression (and high Eomes expression) promotes memory formation (Rao et al., 2010). Exposure to wild-type HSP70i was accompanied by enhanced cytotoxic T cell responses and suppressed responses after exposure to HSP70iQ435A. The same shift toward increased Eomes/T-bet ratios found in mice treated with HSP70iQ435A was also observed in human skin explants. Among cells collected from HSP70iQ435A-treated human skin, Eomes/T-bet ratios were markedly increased. In addition, wild-type HSP70i was shown to up-regulate expression of both CXCR3 and the T cell surface marker CLA implicated in increased homing to vitiligo patient skin observed in earlier studies (Wijngaard et al., 2000).

HSP70i vaccination stimulated humoral responses in mice, directed to the C terminus, leaving the question why naturally occurring antibodies do not deplete HSP70i and prevent the stress protein from inducing autoimmunity. Further analysis of the targeted sequence within HSP70i revealed that circulating antibodies bind downstream of our peptide of interest, offering an explanation for the coexistence of HSP70i overexpression and vitiligo development. Elevated serum levels of HSP70i have, in fact, been reported in coronary artery disease patients, where it was likewise determined that such antibodies do not affect disease expression (Kocsis et al., 2002).

Another vitiligo model included in our studies expresses a transgenic TCR to tyrosinase (Mehrotra et al., 2012). The advantage of including this model is that
spontaneous depigmentation initiates even before animals become available for vaccination. The ears and tail are then fully depigmented, and hair depigmentation initiates by 4 weeks of age. When DNA vaccination was initiated at 5 weeks of age, the pelage remained black yet melanocytes were lost from the hair follicles. Animals untreated or vaccinated with empty vector DNA at that age show a completely depigmented pelage after the hair has regrown. However, in animals treated with HSP70Q435A, the pelage returned pigmented; thus, melanocyte precursors must have migrated from the hair bulge to the follicle and differentiated to express tyrosinase, which is both the antigen recognized by the transgenic TCR as well as the enzyme responsible for pigmentation (Watabe et al., 2004).

Using knockout mouse models, we demonstrated that both helper and cytotoxic T cells are required for depigmentation. Moreover, in both sets of vitiligo-prone mice, depigmentation was accompanied by relevant T cell infiltration to the skin. In the Pmel-1 model, CD8+ T cells were observed infiltrating the hair follicles where depigmentation takes place. In the h3TA2 model, we observed infiltration by transgenic TCR-expressing T cells that can react with remaining melanocytes (Mehrotra et al., 2012). In both models, responses were muted by HSP70iQ435A, attesting to its amazing ability to deactivate an ongoing autoimmune response.

Several lines of evidence support a particular function for stress proteins in DC activation. Similarly, an immature phenotype among DCs supports a regulatory function (Diao et al., 2012). Several recent studies suggest that immunizing autoimmune patients with tolerogenic DCs as a means of interfering with immune responses to self-antigens can be successful, and CD11b-expressing myeloid-derived
suppressor cells have been widely implicated in tumor immune suppression (Ioanno et al., 2012; Rezzonico et al., 2000).

Last but not the least, we were able to induce elevated HSP70i expression, both wild-type and mutant, among lymphocytes collected from gene gun–vaccinated human skin. This provides a platform to translate our findings to a human setting; however, this is also a limitation of the study. Whereas this study suggests that gene gun vaccination may be effective in humans as well, current DNA applications in humans do not make use of a gene gun. We have yet to find whether other means of introducing DNA that are more readily supported by the U.S. Food and Drug Administration will show similar results in human skin, for example, tattooing or electroporation (Bolhassani et al., 2011). Also, we do not yet know whether dampening immune responses by skin application of mutant HSP70i will have consequences for other, more desirable immune responses and perhaps affect systemic immunity as well. It is also possible that additional peptides within HSP70i affect DC activation and currently remain undetected, although this will not necessarily affect our proposed use of HSP70iQ435A in a therapeutic setting. Before performing an actual clinical trial, we cannot be certain that the impact observed on immunocyte profiles in human skin will likewise lead to improved skin pigmentation in treated human patients. Such studies can, however, be further designed following the results presented in our current article. Here, we provide evidence for the positive impact of HSP70iQ435A on DC and T cell profiles and show in vivo data demonstrating that HSP70iQ435A can affect depigmentation and repigmentation in mice, strongly suggesting that the same HSP70i variant will
support repigmentation of lesional skin in human patients.

In summary, the current study gives rise to a potential treatment opportunity for vitiligo by introducing HSP70i with a single amino acid modification. Preclinical data obtained in mouse models reflecting the effector phase of the autoimmune depigmentation response support the use of HSP70i\textsubscript{Q435A} in patients. We have demonstrated that HSP70i\textsubscript{Q435A} DNA can be transfected in human skin and can alter the immune profile toward an anti-inflammatory phenotype similar to results observed in preclinical mouse models. The in vitro and in situ data support the potential significance for human vitiligo; however, it remains to be determined whether the same construct will be effective when treating patients with active disease. DNA vaccination has shown limited overall efficacy when applied to human subjects, yet when applied toward dermatologic conditions, the use of electroporation, for example, enables the next move to a clinical trial in perilesional vitiligo skin using existing constructs (Murakami and Sunada, 2011). Should protein or peptide-based constructs ultimately prove more effective than DNA-based applications, the next step will be to define the minimal sequence to exert an effect upon topical application. With the current strategy, the therapeutic value of HSP70i\textsubscript{Q435A} can offer a potential treatment for vitiligo.
CHAPTER VI: GENERAL DISCUSSION

SUMMARY

Vitiligo is disabling to millions of patients around the world, yet the cause still eludes researchers and clinicians. There is ample evidence that supports vitiligo as an autoimmune disorder, but the mechanism leading to melanocyte reactive T cells has not been defined. The impetus of my dissertation was to firmly establish HSP70i as a critical component in mediating this autoimmune response, and identify a means to block HSP70i. In chapter 3, I determined that HSP70i is located within the melanosome, and is in close proximity to melanosomal antigens. In addition, vitiliginous melanocytes secrete more HSP70i in response to chemical-induced stress. These data suggest a mechanism whereby HSP70i binds melanosomal antigens and is secreted from the cell. Once in the extracellular matrix the HSP70i/melanosomal-antigen complexes activate DCs, which initiates an immune response resulting in targeted killing of melanocytes. In chapter 4, I determined that HSP70i is important for vitiligo development after my results demonstrated that Hsp70-1 knockout mice were resistant to melanocyte-antigen induced depigmentation. By contrast, wild-type mice developed robust vitiligo mediated by cytotoxic T cells. We also showed that vaccination with HSP70i alone accelerates depigmentation in vitiligo-prone mice. I also established that HSP70i activates dendritic cells, which can be abrogated with addition of HSP70i-specific antibodies.
In chapter 5, I determined that the immune activating region of HSP70i resides in the C-terminus, specifically in the sequence QPGVLIQVYEG. Importantly, we generated a mutant isoform (HSP70iQ435A) that prevents and reverses vitiligo. Mechanistically, we determined that wild-type HSP70i activates and drives an inflammatory subset of antigen presenting cells, and induces an effector T cell phenotype. By contrast, treatment with HSP70iQ435A induces an opposite, anti-inflammatory phenotype. We also provided evidence that human cells respond to treatment similarly. Ultimately, the data in this dissertation strongly supports a role for HSP70i in mediating an immune response directed towards melanocytes, and identified HSP70iQ435A as a novel agent for treating autoimmune vitiligo.
Key Findings

Chapter III: Preferential secretion of inducible HSP70 by vitiligo melanocytes under stress

- HSP70i is overexpressed in lesional skin from vitiligo patients
- Cell viability is similar between vitiliginous and control melanocytes in response to MBEH or 4-TBP
- HSP70i is in the same cell fractions as melanosomal antigens as determined by density gradient centrifugation and Western blot
- HSP70i resides in melanosomes as determined by electron and confocal microscopy
- Vitiliginous melanosomes contains greater levels of HSP70i, which can be augmented by MBEH
- Vitiliginous melanocytes secrete more HSP70i in response to chemical (MBEH, 4-TBP) induced stress

Table 2. Summary of key findings
Key Findings

Chapter IV: HSP70i is a critical component of the immune response leading to vitiligo

- DNA vaccination with HSP70i and the melanocyte antigen opt-TRP-1 induces depigmentation in wild-type mice
- Depigmentation occurs locally and distal to the vaccination site demonstrating a systemic response
- Hsp70-1, but not Hsp70-2 knockout mice are resistant to developing vitiligo after HSP70i/opt-TRP-1ee/ng vaccination, thus indicating the requirement for HSP70i in vitiligo development
- Vitiligo is mediated by cytotoxic T lymphocytes as determined by IHC and in vivo CTL assays
- Hsp70-1 knockout mice have reduced T cell skin infiltrates and CTL responses towards the vaccinated antigen
- Vaccination with HSP70i alone accelerates depigmentation in vitiligo-prone mice
- HSP70i activates and drives inflammatory CD11b⁺CD11c⁺ dendritic cells in vivo
- HSP70i protein activates mouse dendritic cells in vitro which can be blocked with antibodies (SPA-810) to HSP70i

Table 2. Summary of key findings
Key Findings

Chapter V: Mutant HSP70 reverses autoimmune depigmentation in vitiligo

- Inflammatory CD11b⁺CD11c⁺ antigen presenting cells are more abundant in Vitiligo patient perilesional skin and peripheral blood
- The C-terminus, but not N-terminus of HSP70i is required for inducing mouse vitiligo
- Vaccination with the C-terminus of HSP70i induces a greater depigmentation response than the full length construct
- The C-terminus induces a humoral response to HSP70i in vaccinated mice.
- The dendritic cell activating sequence (QPGVLIQVYEG) of HSP70i is crucial for depigmentation
- Mutating the dendritic cell activating sequence prevents HSP70i from inducing depigmentation
- The commercially available SPA-810 recognizes the HSP70i sequence including the peptide PGVLIQVYEG
- CD4⁺ and CD8 T cells are required for autoimmune vitiligo as determined from knockout mice
- HSP70i_{Q435A} provides both a prophylactic and therapeutic vaccine against vitiligo development

Table 2. Summary of key findings
Key findings

Chapter V continued

- Vaccination with wild-type HSP70i drives an inflammatory response mediated by activated antigen presenting cells and effector T cells.
- Effects of vaccination with HSP70iQ435 are measurable for > 6 months in P-mel1 mice.
- Vaccination with HSP70iQ435 drives an anti-inflammatory response associated with a greater tolerogenic APC compartment, and a decreased inflammatory APC compartment.
- Wild-type HSP70i increases and HSP70iQ435 decreases the number of skin-infiltrating T cells.
- Wild-type and mutant HSP70i drive effector versus quiescent T cells respectively in vaccinated mice, and human tissues as determined by expression of the T cell transcription factors Eomes and T-bet.
- Vitiligo patient perilesional skin and peripheral blood have greater abundance of effector T cells as determined by Eomes/T-bet staining.
- HSP70i protein increases CLA expression in human tissues.
- Wild-type and HSP70iQ435 bind human dendritic cells, resulting in activation and inhibition respectively.
- Vitiligo patient skin presents with more CXCR3 expressing T cells.
- Wild-type and HSP70iQ435 encoding DNA can be expressed in human skin.

Table 2. Summary of key findings
HSP70i and vitiligo etiology

Several theories have emerged discussing the root cause of vitiligo. Part of this discrepancy is due to the observation of symmetrical lesions in non-segmental vitiligo. This has leant credibility to the neural theory in which melanocytes (which are neural crest derived) are sensitive to neural peptides, catecholamines, or the sympathetic nervous system (Le Poole and Boissy, 1997; Gauthier et al., 2003). Melanocytes arise from the neural crest and migrate along dermatomes during development; thus it is thought that symmetrical loss may be attributed to inadequate sympathetic innervation. There is also the defective adhesion hypothesis, which theorizes that melanocytes fail to attach to the epidermal basement membrane (Gauthier et al., 2003). It has been suggested that increased levels of tenascin, an extracellular matrix glycoprotein in the basal membrane prevents melanocytes from adhering to fibronectin (Le Poole et al., 1997). Failure of melanocytes to adhere may result in their loss or inability to repopulate lesional sites (Le Poole et al., 1997). Similar to the neural theory, defective adhesion could induce symmetrical lesions as a result of the Koebner phenomenon, in which exposed areas of the body (e.g., hands and face) are more sensitive to trauma resulting in greater melanocyte death. Although the adhesion and neural theories are interesting, there has been little supporting evidence. For one, tenascin is known to be upregulated in response to inflammation in several tissues including the skin (Ogawa et al., 2005). Thus, rather than a direct cause of vitiligo, increased tenascin
may result from autoimmunity or inflammation which accelerates vitiligo pathogenesis. In addition, the concentration of neural peptides and catecholamines in the skin is not sufficient to induce melanocyte death (Gauthier et al., 2003). Moreover, these theories fail to address the etiology of segmental (unilateral) or occupational (exposure to bleaching agents) vitiligo which can induce lesions distal to the affected areas. Interestingly, occupational vitiligo often leads to symmetrical (generalized) vitiligo (Boissy and Manga, 2004).

Currently, impaired redox status and autoimmunity are the leading theories for the cause of vitiligo. As I will discuss, these theories may not be independent of each other. With the impaired redox theory, melanocytes are thought to die from increased levels of intracellular oxidative stress, most likely produced during melanogenesis (Gauthier et al., 2003). The increased sensitivity to stress may be linked to genetic and cell abnormalities observed in vitiliginous melanocytes. Low catalase activity leading to increased hydrogen peroxide (H$_2$O$_2$) production has been observed in lesional skin and cultured melanocytes (Goth et al., 2004; Hasse et al., 2004). It has also been shown that vitiliginous melanocytes have impaired calcium uptake which is important for maintaining antioxidant activity (Schallreuter-Wood et al., 1996). From these observations, medications such as pseudocatalyase have been developed to inhibit H$_2$O$_2$ and/or ROS (reactive oxygen species) activity. However, treatment efficacy with pseudocatalyase is minimal, with the majority of studies showing no therapeutic effects (Naini et al., 2012). Moreover, in blinded studies where patients receive a combination of narrowband UVB (NBUVB) and
pseudocatylase, researchers suggest it is the NBUVB rather than pseudocatylase that is having a (minimal) treatment effect (Gawkrodger, 2009).

Support for vitiligo as an autoimmune disease is provided by infiltrates of T cells and macrophages described for the skin of actively depigmenting patients (Le Poole et al., 1996; Ongenae et al., 2003). In addition, the detection of circulating autoantibodies towards melanocyte antigens was amongst the first evidence linking vitiligo to autoimmunity (Bor et al., 1969). It has been shown that antibodies to melanocyte antigens are detected in a large proportion of vitiligo, but not healthy individuals (Xie et al., 1999; Laberge et al., 2005). However, it is controversial which melanocyte antigens these antibodies detect, and is suggested that their production is a result of vitiligo pathogenesis (melanocyte death), rather than a direct cause (Xie et al., 1999). This is supported by the fact that many of these antibodies detect intracellular antigens, rendering these antibodies ineffective in eliciting cytotoxicity (Xie et al., 1999). Interestingly, the melanosomal transmembrane protein MelanA (aka, MART-1; melanocyte antigen recognized by T-cells) only induces CTL, but not humoral responses (Waterman et al., 2002). This suggests that autoimmune destruction of melanocytes is mediated by cytotoxic T cells, rather than antibodies.

Family studies have identified mutations in genes associated with autoimmunity such as HLA which defines antigen presentation; moreover, vitiligo melanocytes often contain abnormal gene expression of proteins essential for antigen processing and protein folding (Stromberg et al., 2008; Lerner, 1959). In addition, vitiligo patients often develop other autoimmune disorders such as thyroid disease, pernicious anemia, Addison's disease, and type 1 diabetes mellitus (Picardi
et al., 2003). These and several other autoimmune disorders have been linked with HLA molecules (Alkhateeb et al., 2003). Patients with HLA molecules susceptible to binding autoantigens have higher frequencies of autoreactive T cells in their circulation (Law et al., 2012). Recently, it was shown that vitiligo patients have increased MHCI (HLA-A*33:01, HLA-B*44:03) and MHCII (HLA-DRB1*07:01) allele expression (Singh et al., 2012). These authors suggest that vitiligo is a type 1 immune response orchestrated by CD4+ T cells which drive CTL autoimmune targeting of melanocytes. Interestingly, vitiligo melanocytes express MHCII (Le Poole et al., 1993c) which is typically restricted to APCs. Thus vitiligo melanocytes may aberrantly display self-antigens on MHCII to CD4+ T cells, which subsequently drive a type I immune response.

We believe that vitiligo is due to autoimmune destruction of melanocytes. This is evident from accumulations of infiltrating melanocyte-reactive T cells in patient skin (Le Poole et al., 1993b). These observations are similar to what we have seen in our animal models where skin taken from vitiligo-prone P-mel1 and h3TA2 mice show an abundance of effector T cell infiltrates (chapters 4 and 5). Moreover, I showed that vaccinations with HSP70i increase the abundance of skin infiltrating CTLs resulting in depigmentation.

Genetic studies provide further support for vitiligo as an autoimmune disorder. A genomewide association study found 10 independent susceptibility loci for generalized vitiligo (Jin et al., 2010). In the MHC encoding region, differential genome expression was observed in both the class I (between HLA-A and HLA-HGC9) and class II (between HLA-DRB1 and HLA-DQA1) genes. Outside the MHC
region, differential expression was identified in several genes linked to autoimmune diseases, many of which are associated with vitiligo (Jin et al., 2010). In addition, a high percentage of vitiligo patients have a single amino acid mutation (Leu155>His) in the NLRP1 (NLR family, pyrin domain containing 1) gene which may contribute to pathogenesis (Jin et al., 2007). NLRP1 encodes for caspases 1 and 7 that activate interleukin-1β, which is overexpressed in vitiligo patients (Levandowski et al., 2013). In addition, a polymorphism is associated with the TYR gene (which encodes tyrosinase) in vitiligo patients (Jin et al., 2010). Interestingly, a TYR_Q402R mutation was identified in vitiligo, whereas TYR_R402Q is closely associated with melanoma (Jin et al., 2010). These authors postulate that peptides containing the TYR_Q402R mutation are more efficiently displayed on MHCI, whereas peptides with the TYR_R402Q mutation may fail to be identified by the immune system (Jin et al., 2010). Both NALP-1 and TYR suggest that a single amino acid mutation can have different effects on immune activation, similar to our HSP70i_Q435A variant.

Because vitiligo is initiated and accelerated under stress, HSP70i may be the link to mediating downstream autoimmune responses. We have shown that vitiliginous melanocytes are more sensitive to stress (chapter 3) with higher production and secretion of HSP70i. Because of the similarities in this response with the proposed redox theory, it is likely that these mechanisms work in sync. Thus, it is likely that vitiliginous melanocytes are inherently sensitive to stress. Exposure to stressors (mechanical damage, UV, chemicals, emotional stress) likely imparts a cellular response, inducing an overproduction of ROS and HSPs. Our results indicate that vitiliginous melanocytes respond to ROS by producing and secreting higher
levels of HSP70i (chapter 3), rather than undergoing apoptosis as proposed by the impaired redox theory. This is similar to previously published results showing that H$_2$O$_2$ treatment upregulates production of HSP70i (Jornot et al., 1991).

A pilot study indicated that HSP70 is aberrantly expressed in vitiligo skin (Le Poole et al., 2004). This is in concordance with recently published data that detected greater expression of HSP70 in vitiligo perilesional and lesional skin (Abdou et al., 2013). In that study, antibodies were directed towards both the constitutive and inducible isoforms (Abdou et al., 2013). Here, I showed that inducible HSP70 specifically is overexpressed in perilesional and lesional tissue (chapter 3). We propose that HSP70i is secreted by melanocytes which initiates autoimmune targeting as discussed in HSP70i functions as a chaperone and adjuvant.

**Future directions for HSP70i and vitiligo etiology:**

My results have strengthened the autoimmune theory, and have shown that HSP70i is a critical component in mediating this response. It may be argued that our animal models do not replicate the environment in naturally occurring vitiligo. We have relied on artificial systems, including vaccinating mice with melanocyte antigens, or utilizing genetically modified mice which express melanocyte reactive TCRs. Fortunately, there are other animal models which display naturally occurring vitiligo, with symptoms closely mimicking human vitiligo. The Smyth Line chicken develops spontaneous vitiligo, and mirrors many of the disease traits in human vitiligo including the development of autoimmune linked diseases such as alopecia and thyroid disease, which are commonly associated with human vitiligo (Smyth,
Depigmentation is observed in the feathers, and is caused by melanocyte-antigen reactive T cells. This T cell response is highly aggressive, leaving Smyth Line chickens prone to uveitis and ultimately blindness. Interestingly, genomic studies in Smyth Line chickens have pointed to abnormal expression of HSP70 (Picardo et al., 2010; Shi et al., 2012), and autoantibodies against HSP70i and melanocyte antigens have been detected (Wang and Erf, 2003). In addition to the Smyth Line chicken, the Sinclair miniature swine displays spontaneous regression of melanoma, often followed by vitiligo development (Picardo et al., 2010). Because pigs have similar physiology to humans, they are often used in pre-clinical studies. Thus the Sinclair swine may be an important next step for testing the treatment potential of HSP70i<sub>Q435A</sub>. In addition, it would be interesting to study the effects of HSP70i in these spontaneous animal models using techniques we have already established in mice. For instance, vitiligo development can be observed following overexpression or silencing (knock-out) of HSP70i. Furthermore, the interactions between redox production, HSP70i expression, and autoimmunity can be explored in more detail. Data from these proposed studies may finally unify several vitiligo etiology theories.

**HSP70i functions as a chaperone and adjuvant**

HSPs are a class of intracellular chaperones shown to be upregulated in response to stress (Lindquist and Craig, 1988; Mehlen et al., 1996). HSPs are involved in protein folding which maintains cellular integrity, and inhibits apoptosis (Lindquist and Craig, 1988; Mehlen et al., 1996). They also have important immune functions such as their ability to process and cross-present antigens via HLA class I
and II pathways, which may invoke both the innate and adaptive immune systems (Javid, 2007; Srivatava, 2002). There is accumulating evidence that HSPs are involved in cross-presentation at several points along the antigen presentation pathway (Srivastava, 2002). The induction of CD8+ T cells by HSPs has important implications as a protective mechanism; however, this function of binding and presenting antigens to APCs could lead to the generation of antigen specific T-lymphocytes, thus providing the basis for an autoimmune response.

Indeed, there are functional redundancies amongst HSPs. For example, gp96 has also been shown to activate DCs directly and via its chaperoned peptides (Kuppner et al., 2001). This lends the argument that HSP70i may not be exclusively responsible for activating the immune response. It should be noted however that many of these studies were carried out under artificial settings, such as removing the ER retention sequence KDEL in order to induce secretion and capitalize on the adjuvant properties of HSPs (Strbo and Podack, 2008). HSP70i on the other hand is special in that it is secreted by living cells, thus its actions occur naturally in vivo (Mambula et al., 2007). In addition, HSP70i is more effective at eliciting immune responses as compared to other HSPs. For instance, the chemoattraction of DCs to Murine Lewis Lung carcinoma cells (3LL) was impaired after RNA silencing of HSP70, but not HSP60, HSP90, or gp96 of the 3LL cells (Chen et al., 2009). I further demonstrated the unique role of HSP70i in vivo, where I examined the exclusive, causative role of HSP70i in inducing autoimmune depigmentation in mice. Using constitutive and inducible HSP70 knockout mouse models, we showed that the
inducible, but not constitutive isoform of HSP70 is solely responsible for inducing and perpetuating an autoimmune response (chapter 4).

HSP70 is the best understood protein of the HSP family, due in part to the resolution of the crystal and solution structures of its substrate binding domain (Zhu et al., 1996; Stevens et al., 2003). These findings have shown that HSP70 preferentially interacts with unfolded peptides, such as NRLLLTG which was used in a crystallography study (Zhu et al., 1996; Javid, 2007). Interestingly, there is evidence that the peptide side-chains may dictate which HSP70 isoforms (BiP, DnaK, HSP70, HSC70, etc.) bind, due in part to subtle differences in the substrate binding domain (Marcinowski et al., 2013). Moreover, recent studies suggest that HSP70 has an affinity for hydrophobic residues located within β-sheet regions of proteins (Marcinowski et al., 2013). This is congruent with the finding that HSP70 binds the hydrophobic tyrosinase peptide b-GSGHWDFAWPWGSGYMNGTMSQV, which is processed by APCs to YMNGTMSQV and displayed on HLA-A2 (Pandya et al., 2009; Noessner et al., 2002). Not surprisingly, MHCI molecules preferentially display hydrophobic peptides (Huang et al., 2011), and provide further evidence that MHCI/II evolved from HSP70 (Hughes and Nei, 1993).

Although it is not well understood how HSP70 exerts its function in vivo, it has been shown that HSP70 secreted from tumors confer anti-tumor responses via the peptides they chaperone (Srivastava, 2002). Thus, HSP70 can be thought to act as both a chaperone, enhancing the immunogenicity towards its bound peptide, and as a chemokine activating APCs directly. Whereas intracellular HSPs serve a cytoprotective function, upon secretion, the inducible isoform of HSP70 functions as
a chaperokine, eliciting innate and adaptive immune responses (Mambula et al., 2007; Asea, 2006; Johnson and Fleshner, 2006)

The mechanism of HSP70i secretion is not understood, but is thought to be induced by the sympathetic nervous system (Johnson and Fleshner, 2006). Many studies suggest that HSP70i secretion involves a non-canonical pathway similar to the cytokine IL-1b, including entering lysosomes where it may be released in to the cytosol, or through exosomal evagination (Calderwood and Murshid, 2012).

Although there is no secretory signal, there is a correlation with production of Lysosomal-associated membrane protein 1 (LAMP1), further supporting HSP70i secretion by an endolysosomal pathway (Mambula et al., 2007). It has also been suggested that HSP70 is transported to the cell membrane by lipid rafts, which may also form budding vesicles (Broquet et al., 2003). Within melanocytes, HSP70i may be secreted along with melanin/melanosomes that are routed to keratinocytes.

As discussed in chapter 3, there is substantial evidence that melanosomes evolved from lysosomes (Orlow, 1995; Le Poole et al., 1993a; Raposo et al., 2002). There is genetic evidence, similarities in structure and physiology (e.g., pH), and shared enzymes such as LAMP-1 that indicate melanosomes are specialized lysosomes (Orlow, 1995). Intriguingly, similar to lysosomes, melanosomes contain the machinery for antigen processing and presentation (MHCI/II) in addition to several unique and highly antigenic proteins (TRP-1/2, Tyrosinase, gp100, MART-1) (Wang et al., 1999). This may address why melanocytes are specifically targeted in vitiligo.
We have demonstrated that vitiliginous melanocytes secrete higher levels of HSP70i in response to stress (chapter 3). This suggests that in vivo, vitiligo melanocytes are more prone to eliciting immune activation, subsequently resulting in their destruction. Under stressful conditions, melanocytes may secrete HSP70i which then activates DCs directly through specific receptor binding, leading to the production of various cytokines such as IL-12, and IFN-γ. In addition, secreted HSP70i may serve as a chaperone, binding melanocytic antigens to be processed by APCs and eliciting autoimmune responses to melanocytes in active disease. DCs activated by HSP70i/melanocyte antigen complexes could lead to the activation and recruitment of effector cells from draining lymph nodes (Srivastava, 2002).

In chapter 3, we showed that HSP70i is in close proximity to melanocyte antigens, as demonstrated by microscopy and Western blotting. It has been demonstrated that HSP70i isolated from melanoma is bound to melanocyte antigens. This is likely to occur in vitiliginous melanocytes, especially considering there are mutations in melanocyte proteins, and abnormalities in the ER (endoplasmic reticulum) and melanosomes (Jin et al., 2010; Le Poole et al., 2000; Li et al., 2009; Boissy et al., 1991). Moreover, we showed that vitiliginous melanocytes have greater colocalization of HSP70i and melanocyte-antigens which is augmented after exposure to MBEH. As mentioned, melanosomes contain MHCII; thus HSP70i is in a position to cross-present peptides. Together, our findings (chapter 3) have determined that HSP70i colocalizes with melanocyte antigens which may be secreted in response to stress. Because of the adjuvant properties of HSP70i, this
may establish a mechanism for autoimmune targeting of melanocytes in vitiligo patients.

**Future directions for HSP70i functions as a chaperone and adjuvant:**

My hypothesis is that HSP70i specifically is required for vitiligo development, due in part to its unique secretion properties. We have shown that HSP70i is secreted by vitiliginous melanocytes, and that it is critical for inducing autoimmune vitiligo. However, it has been shown that HSP70i (and other HSPs) are released by necrosis, but not apoptosis (excluding HSP70i), gaining access to the extracellular matrix (Basu et al., 2000). This suggests that after necrosis, additional HSPs may contribute to immune activation. We demonstrated that knocking out HSP70i in mice prevents the development of vitiligo, yet we did not test whether additional HSPs released by necrotic melanocytes can trigger depigmentation. To test this, we can expose Hsp70-1 knockout mice to 4-TBP (which induces necrosis) or MBEH (which induces apoptosis) to determine if other HSPs can induce immune activation/depigmentation once they have access to APCs. In addition, we can vaccinate C57BL/6 and hsp70-1 knockout mice with melanocyte antigens in combination with alternative HSP isoforms (BiP, DnaK, HSP60/90/110, etc.). Because of the adjuvant/chaperone properties of these HSPs, I would predict depigmentation to occur. However, we have shown that HSP70i is required for depigmentation in naturally developing vitiligo. Determining the adjuvant properties of additional HSPs may be important for developing vaccines towards vitiligo and melanoma.
In addition, we determined that HSP70i is over-secreted by vitiliginous melanocytes, yet did not test whether this secreted HSP70i can activate DCs *in vitro*. It is possible that HSP70i secreted by vitiliginous melanocytes is more antigenic, as may be attributed to having greater access to melanosomal compartments. As such, I have shown that HSP70i is located within melanosomes, yet I did not test whether it is bound to melanocyte antigens; however, others have confirmed this in melanoma (Noessner et al., 2002). This is an important part of our mechanism as it explains the targeted response towards melanocytes. In order to elucidate whether HSP70i is bound to melanocytes antigens, we can use immunoprecipitate assays, and/or fluorescence energy transfer (FRET). Likewise, due to the stress-sensitive nature of vitiliginous melanocytes, proteins are commonly misfolded and/or degraded, which may allow HSP70i to bind peptides not normally accessible in healthy melanocytes. These additional studies will be important for defining HSP70i as a critical adjuvant in vitiligo development.

**HSP70i and immune activation**

Dendritic cells are recognized as one of the most important APC in inducing an immune response. Various HSPs including HSP60, HSP70, and gp96 have been shown to upregulate DC surface expression of MHCII and B7 (CD80/83/86) molecules (Kuppner et al., 2001; Basu et al., 2000). As mentioned, HSP70i is the most abundantly expressed HSP, and is unique in that it can be secreted by live cells of neuronal origin (Georgopoulos and Welch, 1993; Asea, 2007). However, various HSPs with immune activation properties can gain access to APCs after cellular
necrosis. Yet it was shown that silencing HSP70, but not HSP60, HSP90, or gp96 can impair the chemoattraction of DCs to tumor cells (Chen et al., 2009). Thus HSP70i may play a larger role in immune activation. This same study also suggests that HSP70 activates DCs through TLR4 (Toll-like receptor 4), although other reports have suggested the involvement of additional receptors such as TLR2, CD40, and CD14 (Chen et al., 2009, Asea et al., 2002, Asea et al., 2000).

The immune activating region of HSP70i is controversial, with studies suggesting that the N-terminal ATP binding domain as a critical component (Nicchitta, 2003). However, other studies have shown that the C-terminal portion of HSP70 (residues 359-610) activates DCs and other innate cells to produce TNF-α, IL-12, nitric oxide (NO), and drive TH1 responses (Wang et al., 2005; Lehner et al., 2004; Manucha and Valles, 2008; Lehner et al., 2000). In these studies, we have shown that vaccinating mice with the N-terminus of HSP70i has no effect, whereas the C-terminus was critical for inducing depigmentation (chapter 5). In fact, vaccination with the C-terminus gave a stronger response than full length HSP70i.

Working with microbial HSP70 (DnaK), a region that binds and activates DCs was identified (Wang et al., 2005). From this work, and studies that identified cross-recognition between mammalian and microbial HSP leading to autoimmune disorders (Kaufman, 1990), we hypothesized that intrinsic human HSP70i will similarly activate DCs, and that the binding region in human HSP70i can be predicted by aligning human and microbial HSP70. We thus identified the peptide sequence QPGVLIQVYEGER (HSP70_{435-447}) as a potential target for blocking entities to prevent DC activation (patent application PCT/US2008/076266).
Because vaccinations with peptides have proven an effective means to induce immune responses and generate antibodies towards the full length proteins (Berzovsky, 1995), we predicted the peptide sequence QPGVLIQVYEGER as a novel target in preventing an autoimmune response. The importance of the DC activating peptide lies in opportunities to bind and prevent its association with specific receptors. Because of the promiscuity of HSP70 in binding inflammatory receptors, finding blocking agents against HSP70i will be a more achievable task in inhibiting APC activation. In addition, it will avoid non-specific activation by these receptors. Our findings suggest that blocking this peptide with antibodies (e.g., SPA-810) can prevent DC activation (chapter 4). These results may prove useful as a treatment against autoimmune disorders, or alleviate inflammation. Unfortunately, SPA-810 did give some unwanted DC activation, most likely attributed to Fc receptor binding. Developing a Fab version of SPA-810 may alleviate these unwanted responses.

As mentioned, residues 407-426 in DnaK were identified to enhance DC maturation, and induce the production of IL-12 (Wang et al., 2005). BLAST analysis of this peptide region recognizes the sequence QPGVLIQVYEGERAMTKDNN to be critical for human DC activation, of which we identified QPGVLIQVYEGER as being homologous to DnaK. However, it should be noted that this group identified an additional peptide, IVHVTAKDKGTGKENTIRIQEGSGLSKEDIDRMKDAEH, located downstream of the peptide binding groove which may have immune inhibitory properties. A BLAST search identifies ILNVTATDKSTGKANKITITNDKGRSKEEIRERMVQEAEKY, with 54% homology in mammalian HSP70i. This is interesting as there has been controversy regarding
HSP70i in immune activation. Some studies suggest that HSP70 is important for inhibiting inflammation, and has tolerating effects toward self antigens mediated by DCs in other disease models, although the mechanism was not defined (Millar et al., 2003). It is also suggested that endotoxin contamination of HSP70 contributes to DC activation in vitro (Stocki et al., 2012). It should be noted that we achieved DC activation with endotoxin-free HSP70i protein, as well as gene-gun vaccination with DNA-encoding plasmids.

It may be that HSP70i has activating or inhibitory functions depending on the peptide it is bound to, or the TLRs it binds. Having both activating and inhibitory sequences could allow HSP70i to have “on” and “off” properties which may be dictated by early and late stages of inflammation or infection. As mentioned, HSP70i may bind several TLRs including CD91, TLR-2, CD14/TLR4, CCR5, and several scavenger receptors (Thériault et al., 2006; Aneja et al., 2006; Floto et al., 2006; Qazi et al., 2007; Fischer et al., 2010). Depending on the receptor(s) HSP70i binds, cell signaling is thought to occur through mNF-κB, and/or the MAP-kinase family (ERK1/2, p38, and SAPK/JNK) (Aliprantis et al., 2002; Oppermann et al., 2004). In fact, DCs express different cytokines depending on which TLR(s) are agonized, including the production of inhibitory cytokine IL-10 (Mayer et al., 2009). Thus, HSP70i may induce DC inhibition or activation depending on the TLR it binds. Studies also indicate that binding to surface CLR (C-type lectin receptors) results in DC inhibition (Meyer-Wentrup et al., 2008). Moreover, DCs can be induced to provide inhibitory signals to T cells directly (e.g., signaling through CTLA-4 and PD-1) or secrete IL-10 to upregulate production of Tregs (Lau et al., 2008). This may
provide a mechanism whereby wild-type and HSP70i_Q435A bind a different repertoire of TLRs/CLRs, resulting in opposite responses attributed to these proteins.

We have further shown the importance of immune activation by HSP70i in vivo. The vitiligo prone mouse strain Pmel-1 faithfully mimic human vitiligo in which melanocyte-reactive T cells escape clonal deletion, and stress is shown to augment the T cell response (Overwijk et al., 2003). We chose to vaccinate these mice with mutant HSP70i_Q435A to test whether a mutation at residue 435 would have an effect on depigmentation, since this mutation prevented SPA-810 Ab from binding. Here, we showed that depigmentation is accelerated after vaccination with wild-type HSP70i, whereas mutant HSP70i_Q435A slowed down the rate of depigmentation (chapter 5). This may indicate that HSP70i_Q435A induces an antagonistic effect when binding to DCs. Alternatively, autoantibodies may be generated against HSP70i through molecular mimicry as has been previously observed with bacterial infection (Kaufmann, 1990). My results demonstrated that wild-type and HSP70i_Q435A have opposite effects on DC activation. As a result of this, downstream T cell activation is likely affected. We showed that splenocytes and skin from wild-type and HSP70i_Q435A vaccinated mice display effector versus quiescent phenotypes as determined by EOMES and T-bet ratios. Thus, it is possible that HSP70i_Q435A is acting as a dominant-negative, or is binding a different repertoire of TLRs leading to suppressed immunity.
Future directions for HSP70i and immune activation:

Although the studies with wild-type and HSP70i_{Q435A} have shown astounding results, the mechanism of action is still not fully understood. The response elicited by APCs depends on which TLRs are agonized, or whether HSPs are taken up through a non-receptor route (Calderwood et al., 2012). In addition, the peptides that are bound by HSPs may induce immune activation, such as anti-tumor stimulation through antigen-cross presentation. It is possible that peptide binding may be affected in HSP70i_{Q435A}, although this is not likely as the mutation lies outside the substrate binding cleft. As mentioned, HSP70i_{Q435A} may bind a different subset of TLRs leading to inhibition. Alternatively, HSP70i_{Q435A} may bind the same TLRs as wild-type HSP70i without causing activation. In this regard, HSP70i_{Q435A} may be acting as an antagonist, or dominant negative. In either case, determining receptor binding and resulting cell signaling of HSP70i_{Q435A} will be crucial for understanding its immune effects. Interestingly, HSPs can be taken up by non-APCs as well. It was shown that HSP90 can be taken up by Chinese Hamster Ovary cells by scavenger receptor binding, where it is transported to proteasomes and interacts with antigens (Calderwood et al., 2012). It would be interesting to test whether melanocytes can take up wild-type and/or mutant HSP70i.

From studies using DnaK we hypothesized that the DC activating region would be homologous to human HSP70i. Through a database search, we identified and patented the peptide sequence QPGVLIQVYEGER (HSP70_{435-447}). Here, we show that as little as one residue change in this region is sufficient to decrease depigmentation in mice when vaccinated in combination with TRP-2. Because our
goal is to bring HSP70i vaccinations to the clinic, DnaK may provide an even stronger immune response as compared to mammalian HSP70i. Likewise, studies have identified cross-recognition between mammalian and microbial HSP leading to autoimmune disorders (Kauffman, 1990). Thus, vaccination with DnaK may induce mimicry in which the immune system targets and neutralizes HSP70i from activating an immune response.

**HSP70i and autoimmunity/tumor immunity**

Observations that HSPs can serve as adjuvants in anti-tumor vaccines first suggested to us a link between these proteins and vitiligo. Melanocytic antigens in combination with HSP70 have been shown to elicit immune responses towards cancerous and healthy melanocytes (Das et al., 2001; Castelli et al., 2004). As with the anti-tumor vaccines, secreted HSP70i can be thought of as a chaperokine, binding melanocytic antigens which can be processed by APCs (Javid et al., 2007; Srivastava, 2002). The relationship between HSPs and melanocytic killing suggest a role for HSP70i in mediating stress induced events that elicit auto-immune responses in vitiligo.

Targeting tumors with self-antigens has been en vogue for several years (Castelli et al., 2004). Importantly, it was shown that injecting mice with a combination of HSP70 and melanocyte antigen encoding plasmids is sufficient for inducing vitiligo, and this response is mediated by CD8+ T cells (Sanchez-Perez et al., 2006; Mosenson et al., 2012). This study demonstrates the shared relationship between vitiligo and melanoma in the targeting of specific cells mediated by HSPs.
Here, we showed that depigmentation in C57BL/6 mice occurred within and distal to the site of the gene gun vaccinations (chapters 4 and 5). As with the findings by Denman (2008), cytotoxicity towards melanocytes was associated with an antigen-specific and systemic immune response to melanocyte antigen. Here, Hsp70-1 knockout mice displayed reduced killing of melanocyte antigen pulsed splenocytes, indicating a role for HSP70i in the immunogenicity towards these cells. These results mirror the melanoma studies in which a CTL response was only elicited after inclusion of HSP70 (Sanchez-Perez et al., 2006). In the case of tumor immunity, HSP70i is thought to efficiently present self-antigens, or directly activate the innate immune system (Nicchitta, 2003). In the latter, APCs will be activated leading to the recruitment of effector cells, and the eventual killing of target cells (Nicchitta, 2003).

Previous vaccination studies using HSP70i in combination with melanocyte antigens at suboptimal levels have shown that immune responses can be elicited towards these target antigens (Denman et al., 2008). Depigmentation preferentially occurred after inclusion of HSP70i in the vaccine, for the first time demonstrating the importance of this protein in mediating autoimmune responses to melanocytes (Denman et al., 2008). Tumor studies have shown that directing HSP70 towards melanocytes can enhance anti-melanoma responses (Sanchez-Perez et al., 2006), demonstrating the shared relationship between vitiligo and melanoma in the targeting of specific cells mediated by HSPs. In the current studies, reduced killing of melanocyte antigen-pulsed splenocytes was observed in hsp70-1 knockout mice, providing further support for a role of HSP70 in the immunogenicity towards these cells.
In addition to vitiligo, HSPs have been implicated in several other autoimmune disorders including psoriasis, autoimmune intestinal inflammation, autoimmune encephalomyelitis, and Kawasaki disease (Seung et al., 2007; Steinhoff et al., 1999; Mor and Cohen, 1992; Jin et al., 2007). Although the mechanisms for the etiology of these diseases remains elusive, HSPs are thought to initiate an inflammatory response which may cause damage to susceptible tissues, such as the bowel. Innate cells activated by HSPs are known to secrete several inflammatory cytokines including IL-6, IL-12, and TNFα contributing to tissue damage (Asea et al., 2007). In addition to systemic inflammation, peptides bound to these HSPs may induce cell targeting after processing by APCs.

By contrast, other studies have shown that HSPs may in fact play a protective role in preventing autoimmunity. HSP65 for instance has been shown to treat autoimmune arthritis in Lewis rats (Rajaiah and Moudgil, 2009). It is thought that HSP65 (as well as other HSPs including HSP70) activates Tregs which suppress the autoimmune response (Rajaiah and Moudgil, 2009; Borges et al., 2012). Moreover, several autoimmune disorders including arthritis, atherosclerosis, and diabetes have been associated with antibody targeting of HSPs (Rajaiah and Moudgil, 2009). It is thought that infections such as Mycobacterium tuberculosis induce the production of antibodies towards bacterial antigens including HSPs. Because of the evolutionally conserved nature of these proteins, mimicry may occur in which the antibodies target mammalian HSPs. It is thought that these antibodies bind and damage cells expressing surface HSPs, such as endothelial cells (Rajaiah and Moudgil, 2009). Together, the role of HSPs in autoimmunity remains controversial,
with evidence for both a protective and pathogenic role. This dichotomy may explain my results in which wild-type HSP70i is important for initiating autoimmune vitiligo, whereas HSP70i\textsubscript{Q435A} inhibits this response. Whether the effects are due to differential processing, receptor binding, or antibody production remains to be determined.

In conclusion, we have shown that HSP70i is necessary and sufficient to precipitate vitiligo. Importantly, mutant HSP70i\textsubscript{Q435A}-encoding DNA was able to reverse the inflammatory phenotype of DCs, prevent infiltration of melanocyte reactive T cells to the skin, and avert depigmentation. The general strategy is outlined in Figure 33. This leaves several important and interesting questions to be addressed. Can human patients be treated with a DNA vaccine introduced to the skin? Will it affect the development of other autoimmune diseases, or cancer? The responses HSP70i will ignite are directed towards the antigens chaperoned by the stress protein. Thus, muted responses may likewise depend on the peptides bound to the (mutant) stress protein, and may be less dependent on the stress protein (if any) engaged in disease precipitation. When stimulating anti-tumor responses or interfering with responses to self, such substrate specificity or the source of the heat shock protein can help selectively support therapeutic effects. Another selective approach makes use of the exclusive surface expression of HSP70 family members by tumors. This renders the extracellular portion amenable to targeting by antibodies, and antibodies to HSP70 can be therapeutic in cancer (Stangl et al., 2011).
Taken together, targeting HSP70i is a promising approach towards the treatment of vitiligo. Mutant HSP70iQ435A prevented the depigmentation process in different mouse models prone to vitiligo development. Moreover, HSP70iQ435A reversed vitiligo as was seen in h3TA2 mice, where active depigmentation was occurring. We believe that HSP70iQ435A dampens the autoimmune response, allowing melanocyte stem cells to repopulate affected areas. Future studies will show how best to translate findings to a clinical trial to determine the safety and efficacy of mutant HSP70 treatment in vitiligo patients.

Future directions for HSP70i and autoimmunity/tumor immunity:

Bringing HSP70iQ435A to the clinic will be the ultimate goal. Because of the contradictory results regarding HSPs and the immune system (pathogenic versus protective) understanding how wild-type and mutant HSP70i function will need to be addressed. This will include understanding whether there is uptake and presentation by APCs, identifying the TLRs and CLRs each of these proteins bind, and the role that HSP70i antibodies play in mediating inflammatory versus anti-inflammatory immune responses.

We have shown that blocking HSP70i and vaccination with HSP70iQ435A are viable methods for treating vitiligo. It will be important to test these therapies in other autoimmune disorders. HSPs are implicated in several autoimmune disorders, of which animals models have been developed. We have chosen administration of DNA in the skin by gene gun vaccination as this allows the protein to be expressed locally in the affected sites. It may be necessary to modify the route of
administration depending on the disease. This may include intramuscular or oral
administration. Moreover, because gene gun vaccinations are not fully approved for
the clinic, we will need to test alternate methods. This may include DNA
electroporation, or even tattooing, both of which are FDA approved in other
settings. Also, it may be necessary to use RNA or protein instead of DNA. Modifying
the vector to include shorter peptides (such as the DC activating region) may prove
more effective than full length protein, similar to our finding in chapter 5. In
addition, it may be beneficial to treat patients with lenti/retrovirus carrying the
HSP70iQ435A gene. These viruses can be modified to allow for stable infection, thus
eliminating the need for booster vaccinations. In addition, the viruses can encode
ligands/receptors to bind specific cell types (e.g., DCs, melanocytes, etc), and may
allow for a local (skin) versus systemic response.

In addition to treating vitiligo, HSP70iQ435A may provide a treatment for
melanoma. These diseases are considered “mirror images” in that vitiligo leads to
autoimmune targeting of melanocytes, whereas melanoma is an overproliferation of
these cells that often avoid immune detection. Although vitiligo and melanoma
develop by different mechanisms, there may be shared therapeutic targets (Stangl et
al, 2010). Thus, vaccination with HSP70iQ435A may be the first treatment to achieve
anti-tumor responses without the induction of autoimmunity.
CHAPTER VII: GENERAL METHODS

Mice

C57BL/6, B6.129S2-Cd4tm1Mak/J (CD4 knockout), B6.129S2-Cd8atm1Mak/J (CD8 knockout), and the vitiligo-prone 78B6.Cg-Thy1a/CyTg(TcraTcrb)8Rest/J (Pmel-1) gp100-reactive TCR transgenic mice were purchased from Jackson Laboratories. Inducible hsp70 (Hsp70-1) knockout mice (B6;129S7-Hspa1a / Hspa1btm1Dix / Mmcd) were purchased from Mutant Mouse Resource Center (UC Davis, CA, USA). Constitutive hsp70 (Hsp70-2) knockout mice were contributed by Dr. E.M. Eddy and maintained as heterozygotes; male Hsp70-2 knockout mice are infertile, whereas females are not (Eddy, 1998). A neomycin insert precedes the Hsp70-2 gene; DNA was isolated from mice tail snips, followed by PCR with the appropriate primers to determine homozygosity of the offspring with Hsp70-2 knockout mice displaying the presence of neomycin gene insert that obliterates Hsp70-2 expression and complete absence of Hsp70-2-encoding DNA. Wild-type mice were also selected and used in the Hsp70-2 knockout experiments. Vitiligo-prone h3TA2 tyrosinase-reactive TCR transgenic mice were generated in the laboratory of S.M. In Pmel-1 mice, the melanocyte-reactive, transgenic TCR is CD8-dependent and is thus expressed on most of the CD8+ T cells. In these mice, relevant T cells are followed by virtue of CD8 expression. In h3TA2 mice, however, the major histocompatibility
complex class I–restricted, transgenic TCR was originally isolated from human CD4+ tumor-infiltrating lymphocytes and functions independent of CD8 co-receptor expression. Most of the transgenic T cells are CD4−CD8− in this model (Mehrotra et al., 2012). In these mice, transgenic T cells were followed by CD3 expression and detection of the TCR transgene. Mice were 5 to 8 weeks old when included in experiments, excluding the Hsp70-2 knockouts and associated control animals that were 18–24 months of age. Animals were gender-matched to wild-type controls where relevant. Experiments included 10 mice per group and are repeated twice unless stated otherwise in the figure legends. Mice were maintained in facilities approved by Loyola University Institutional Animal Care and Use Committee regulations and adhered to guidelines provided by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

**Human tissues**

Blood samples (30 ml) were obtained from patients with active vitiligo, defined as noticeable progression over the past 3 months. PBMCs were purified over a Ficoll density gradient, washed in phosphate-buffered saline, and stored in 10% dimethyl sulfoxide under liquid nitrogen until further use. Four-millimeter punch biopsies were obtained from the border of actively progressing vitiligo lesions in volunteer patients attending the Loyola Dermatology outpatient clinic or from patients undergoing unrelated surgeries at Rush University Medical Center. Tissues and blood samples were obtained from three or more patients per group, and staining was performed on 10,000 cells per fluorocytometry sample. Immunohistochemical
analysis was performed at least in triplicate for each staining and on each tissue sample, on at least three sections for each staining. Actual sample sizes are listed in the figure legends. Informed consent was obtained from patients, and all samples were obtained with approval from the Institutional Review Board at Loyola University and at Rush University Medical Center, adopting the principles described in the Declaration of Helsinki.

**Preparation of bleaching agents**

4-tertiary butyl phenol 4-TBP (Sigma, St. Louis, Missouri, USA) was prepared as a stock solution of 250 millimol in 70% ethanol and diluted to a working concentration of 250 μmol. Monobenzyl ether of hydroquinone MBEH (Sigma) was dissolved in 20% dimethyl sulfoxide (Sigma) and mixed with 70% ethanol for a stock concentration of 250 millimol. 4-TBP and MBEH were diluted with media to the listed working concentrations.

**Cell Culture**

For all experiments using melanocytes, cells were harvested from skin incubated overnight in (0.1 mg/ml trypsin (Mediatech, Manassas, VA, USA), 100 IU/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml amphotericin (Mediatech) prepared in Dulbecco’s Phosphate Buffered Solution (DPBS; Mediatech). Melanocytes were maintained in melanocyte media: Ham's F-12 medium (Mediatech) with 2 mmol/l glutamine (Mediatech), 100 IU/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml amphotericin (Mediatech), 0.1 mmol/l isobutylmethylxanthine (Sigma, St. Louis,
Missouri, USA), 10 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (Sigma), and 1% Ultroser G serum substitute (PALL Life Sciences, Port Washington, NY, USA). Informed consent was obtained from patients and all samples were obtained with approval from the Institutional Review Board at Loyola University, adopting the principles described in the Declaration of Helsinki.

**Melanocyte Fractions**

HM162P7 melanocytes were dounced with a tight pestle (pestle 'A'; Pierce, Rockford, IL, USA) on ice in hypotonic buffer presence of antipain, leupeptin, phosphoramidon, pefabloc and aprotinin before underlayering a 5-30% iodixanol gradient then spun for 18 hrs at 29,000 rpm. Resulting fractions were collected from the top.

**Western Blotting**

Cell lysates were obtained from healthy melanocytes (HM162, p7) and protein content was measured using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA), for equal loading at 20 μl for cellular proteins, or 5 μg of purified HSP70 per slot onto a 10% polyacrylamide minigel. After electrophoresis, separated proteins were transferred on to Immobilon-P membrane (Millipore, Billerica, MA, USA). The blots were incubated with SPA-820 to constitutive and inducible HSP70 (mouse monoclonal IgG1; Enzo Life Sciences). In a separate experiment blots were incubated with SPA-811 to inducible HSP70 (polyclonal rabbit; Enzo Life Sciences), and HMB45 to gp100 (monoclonal mouse IgG1; Dako, Carpinteria, CA, USA). After
washing, blots were reacted with horseradish peroxidase labeled goat anti-mouse
antiserum; Dako) or alkaline phosphatase labeled goat anti-rabbit antiserum Dako)
conjugated secondary Abs. Blots were developed with amino ethyl carbazole
(Sigma) as the substrate.

**Electron Microscopy**

After cell fractionation, HM162P7 fractions (7-9 and 20-22) were pooled then
centrifuged at 10,000rpm for 10 minutes to pellet the cellular material. Gradient
material was aspirated off and the cellular material re-suspended in 500ul of half-
strength Karnovski’s fixative for 30 minutes at room temperature. Material was re-
centrifuged, fixative aspirated, 1 ml of 1% Agar in sodium cacodylate buffer added
(Agar, low melting, SIGMA cat#A-5030) and refrigerated overnight. The Agar
gelatin cast containing the pellet was processed in EPON 812 (Ted Pella, Inc.,
Redding, CA) by routine procedure (Boissy et al., 1991). For immunocytochemistry,
melanocytes were cultured in Tissue-Tek chamber slides and treated with 4-TBP as
previously described. Cells were then washed, fixed with half-strength Karnovski’s
fixative, and processed in EPON 812 (Ted Pella, Inc.) by routine procedure. Sections
were obtained using a RMC-MT6000XL ultramicrotome and stained with uranyl
acetate and lead citrate. For immunocytochemistry, sections were washed, treated
with mouse anti-HSP70i at 1:5, wash, treated with goat anti-mouse antibody
conjugated to 5 nm gold particles (Ted Pella, Inc.) at 1:10, and counterfixed and
stained by routine procedures. All sections were viewed, and selected images were
digitally photographed using a JEOL JEM-1230 transmission electron microscope.
**Cytoxicity assay**

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays (Bioassay system, Hayward, California, USA) according to the manufacturer's instructions. Briefly, 20,000 cells/well were plated in triplicate wells of a 96-well plate to attach overnight. Cells were either treated with vehicle alone or with 125, 250, or 500 μM 4-TBP or MBEH for 72 h. MTT reagent (tetrazole) was added to the cells and incubated in a 37ºC humidified chamber for 4 h. Tetrazole is converted to formazan in the mitochondria of living cells. The formazan crystals formed were solubilized in buffer (PBS) and the wavelength was read in a reader at 562 nm (BMG Labtech, Inc., Durham, North Carolina, USA). Cell viability was calculated as absorbance in % of the vehicle-treated control. Primary melanocytes: healthy neonatal (Mf0887P2; Mf0627P6; Mf0644P1; Mf0865P4; Mf0862P2; Mf1005P5; Mf0880P2), healthy adult (Mu09236P3), and adult vitiligo (Mc10103P4; Mc1054P2; Mc1043P2; Mu08114P7; Mc1044 P4).

**HSP70i ELISA**

Melanocytes from vitiligo and non-vitiligo donors were plated in triplicate at 20,000 cells per well, and the immortalized cell lines PIG1 and PIG3V were plated in triplicate at 10,000 cells per well in a 96 well flat-bottom plate (Falcon). Cells were treated for 24 hours with either 125 μmol MBEH (prepared as above), vehicle (20% dimethyl sulfoxide diluted in 70% EtOH, and diluted to 1:1000 in melanocyte media), or media alone. All cells were maintained in a total volume of 150 μl. After 24 hours, cells were imaged, and 100 μl of supernatant was added to an HSP70i high
sensitivity ELISA kit (Enzo Life Sciences) and HSP70i was detected according to manufactures instructions. Primary melanocytes: healthy adult melanocytes from Invitrogen (HEMa-LP C024-5C; lot# 5C061P6; lot# 1183797P3; Life Technologies, Carlsbad, CA, USA), healthy neonatal (Mf12385P5; Mf12387P5) and adult vitiligo (Mc1044P6; Ma13090P1; Mp12134P4). Immortalized cell lines: healthy PIG1 P96 and vitiligo PIG3VP54.

Confocal microscopy

Melanocytes isolated from healthy neonatal foreskin, or adult vitiligo skin were plated at 10,000 cells per well on 8 well gelatin coated Lab-Tek chamber slides (Thermo Fisher Scientific, Rochester, NY, USA). Cells were maintained in melanocyte media as described above. Cells were treated for 24 hours with 250 μM of either 4-TBP, MBEH, or untreated. The cells were fixed with 3.7% formaldehyde (Polysciences) in 0.1 M PIPES, pH 6.8 [piperazine-N, N'-bis (2-ethanesulfonic acid)] (Sigma). Polyclonal rabbit anti-HSP72 (SPA-811; Enzo Life Sciences) and monoclonal mouse anti-gp100 (HMB45; Dako) were incubated in 0.3% saponin (Sigma) in PBS. After washing, fluorescent staining was performed with FITC conjugated goat anti-rabbit, and PE/Cy7 goat anti-mouse secondary antibodies (Southern Biotech). Confocal images were captured using a Zeiss LSM 510 scanning electron microscope (Zeiss, Maple Grove, MN, USA). Image analysis was performed using Adobe Photoshop (Adobe Systems Inc.) and ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA) software. For visualizing colocalization, the following channels were used: FITC as green, and PE/Cy7 as red. Colocalization was quantified
with the ImageJ plug-in JACoP (Just Another Co-localization Plugin; Bolte and Cordelières, 2006). Primary melanocytes: healthy neonatal (Mf0887P7; Mf0627P4; Mf11234P1) and adult vitiligo (Mu0885P11; Mc1044P11; Mu0885 P4).

Immunohistology

Mouse tissue: After euthanasia, mouse skin biopsies from vaccinated sites were snap-frozen in OCT compound (Sakura Finetek). Cryostat sections (8 μm) were fixed in cold acetone, and indirect immunoperoxidase staining procedures were performed essentially as described previously (Le Poole et al., 1993b). Tissue sections were treated with Super Block (ScyTek) to prevent nonspecific antibody binding. Primary antibodies Ta99 to mouse TRP-1 (mouse monoclonal; Covance), biotinylated 145-2C11 to CD3ε (Armenian hamster monoclonal; BD Pharmingen), biotinylated 53-6.7 to CD8α (mouse monoclonal; BioLegend), and 511 to Vβ12 (mouse monoclonal; Thermo Scientific) followed by horseradish peroxidase–conjugated secondary antibodies [streptavidin, goat anti-mouse immunoglobulin 2a (IgG2a), or goat anti-rat; Santa Cruz Biotechnology Inc.] were used. Enzymatic detection was finalized with aminoethylcarbazole as a substrate (Sigma). Staining was quantified as the number of cells per square millimeter of skin observed by light microscopy (Nikon) and Adobe Photoshop software (Adobe Systems Inc.). Fluorescent staining was performed with APC/Cy7-conjugated 145-2C11 to mouse CD3ε (Biolegend), fluorescein isothiocyanate (FITC)-conjugated 536.7 to mouse CD8α (BD Pharmingen), and V450-conjugated RM4-5 to mouse CD4 (BD Pharmingen). Confocal images were captured using a Zeiss LSM 510 scanning
electron microscope (Zeiss, Maple Grove, MN, USA). Image analysis was performed using Adobe Photoshop (Adobe Systems Inc.) and ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA) software. For visualizing colocalization, the following channels were used: APC / Cy7 visualized as red, FITC as green, and V450 as blue.

*Human tissue:* Human skin sections were prepared as above. Four-millimeter punch biopsies were obtained from non-vitiligo control patients, or non-lesional and lesional areas of actively progressing vitiligo in volunteer patients attending the Loyola Dermatology outpatient clinic. Primary antibodies EP1345Y to human CD11b (rabbit monoclonal; Novus Biologicals), B-ly6 to CD11c (mouse monoclonal; BD Pharmingen), 16H4L5 to T-bet (rabbit monoclonal; Invitrogen), rabbit serum to Eomes452–618 (rabbit polyclonal; Novus Biologicals), 49801 to CXCR3 (mouse monoclonal; R&D Systems), and SPA-810 to HSP70i (mouse monoclonal; Enzo Life Sciences formerly Stressgen), followed by horseradish peroxidase–conjugated secondary antibodies (streptavidin, goat anti-mouse IgG2a, or goat anti-rabbit; Santa Cruz Biotechnology Inc.). Enzymatic detection and quantification was performed as described above. Immunohistochemical analysis was performed at least in triplicate for each staining and on each tissue sample, on at least three sections for each staining. Actual sample sizes are listed in the figure legends. Informed consent was obtained from patients, and all samples were obtained with approval from the Institutional Review Board at Loyola University, adopting the principles described in the Declaration of Helsinki.
Activating immature DCs and in vitro cell cultures

**Human DCs:** Generation of DCs from human PBMCs was prepared from a modified protocol (27). Briefly, PBMCs were isolated on different occasions from whole blood of healthy volunteers by Ficoll-Paque (GE Healthcare) density gradient centrifugation. The experiment was performed three times with samples from different donors. Subsequently, monocytes were enriched with the EasySep Human Monocyte Enrichment Kit (Stemcell Technologies) according to the manufacturer’s instructions. Data from a representative experiment are shown. To generate DCs, we maintained the cells in Teflon containers (Savillex) to prevent adherence in RPMI medium (Mediatech Inc.) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), recombinant human GM-CSF (100 ng/ml) (Leukine, Berlex), and recombinant human IL-4 (25 ng/ml) (R&D Systems). On day 7, either LPS (Sigma) or HSP70i (1 μg/ml) (BioVision) was added. To assess the wild-type and HSP70iQ435A isoforms, we isolated His-tagged protein from transfected COS7 cells with His-Select Spin Columns (Sigma) and added it at a final concentration of 1 μg/ml to the DCs. After 24 hours, expression of activation markers was assessed as described under flow cytometry.

**Mouse DCs:** Bone marrow-derived monocytes were prepared by culturing murine bone marrow cells using a modified protocol (Boudreau et al., 2008). Briefly, bone marrow cells were flushed aseptically from the femurs and the tibia of mice. Monocytes were enriched using the EasySep Mouse Monocyte Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer’s
To generate dendritic cells, non-adherent cells were maintained in Teflon containers (Savillex, Minnetonka, MN, USA), in Roswell Park Memorial Institute medium (RPMI) (Mediatech Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 ng/ml murine GM-CSF (Peprotech, Rocky Hill, NJ, USA), and 12.5 ng/ml murine interleukin-4 (IL-4) (Peprotech). On day 7, 1 μg/ml of either lipopolysaccharide (Sigma) or HSP70i (Biovision, San Francisco, CA, USA) was added. To block HSP70i, the cells were first incubated with 2 μl/ml of Fc-receptor block (rat anti-mouse CD16/CD32; BD Pharmigen) followed by the addition of HSP70i (Biovision) and SPA-810 to HSP70i (mouse monoclonal; Enzo Life Sciences Inc., Farmingdale, NY, USA). After 24 h, the expression of activation markers was assessed as described under flow cytometry.

**Site-directed mutagenesis**

Human HSP70i and microbial DnaK were aligned in a BLAST search to identify a 13–amino acid stretch as the putative DC-activating peptide region within human HSP70. To test this, we next created vectors with single- or double-nucleotide mutations in the putative DC-binding region of human HSP70i (HSP70i435–447) using appropriate primers to induce mutations in the sequence QPGVLIQVYEGER. As a template for the site-directed mutagenesis, our expression vector encoding HSP70i was used as described (12). In vitro mutagenesis of HSP70 residues was performed on a double-stranded template with polymerase chain reaction as previously described (45). Mutant sequences were verified by DNA sequencing (Sequenase...
version 2.0, U.S. Biochemical Corp.). To verify protein expression, we transfected COS7 cells (Invitrogen) with individual plasmids encoding human HSP70i or mutant HSP70i plasmids using Lipofectamine (Invitrogen), and we verified protein expression by Western blotting. Blots were probed with anti-HSP70 antibody (rabbit polyclonal antibody SPA-811 or mouse monoclonal antibody SPA-810; Assay Designs) and horseradish peroxidase–conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse; Santa Cruz Biotechnology Inc.). Blots were developed with aminoethylcarbazole as a substrate (Sigma).

**Gene gun vaccinations**

Vaccinations for all experiments were prepared as described previously (12) unless stated otherwise. Briefly, bullets were prepared by precipitating endotoxin-free plasmid DNA onto spermidine-coated gold beads (Fluka BioChemika and Sigma-Aldrich) and used to coat silicone tubing (Bio-Rad). All bullets were used within 14 days of preparation and maintained under vacuum before use. Mice were prepared for vaccination by biweekly abdominal hair removal with Nair (Church and Dwight Co.) and vaccinated with a Helios Gene Gun (Bio-Rad) under isoflurane anesthesia (E-Z Euthanex gas chamber, E-Z Systems Corp.). Unless otherwise stated, mice were ventrally vaccinated weekly for four consecutive weeks with a 4.8-μg dose of total DNA. Where combinations of plasmids were used, such DNA was mixed 1:1 to the same total dose of DNA and combined to prepare bullets.
Evaluating depigmentation

Depigmentation was quantified by flatbed scanning of the ventral and dorsal sides of mice under anesthesia immediately before the initial vaccination and 4 weeks after final vaccination (6 months after the final vaccination in Pmel-1 mice), and subsequent image analysis was performed with Adobe Photoshop software (Adobe Systems Inc.). The percent depigmentation was calculated from the largest evaluable area as the percentage of pixels among >150,000 evaluated with a luminosity above the cutoff level set to include 95% of pixels for untreated mice (12). Mice were also photographed with a DSC-S950 SteadyShot digital camera (Sony) as indicated.

Assessment of CTL activation

In vivo cytotoxicity assays were performed as described (Denman et al., 2008). Briefly, 4 weeks after evaluating depigmentation, mice received two booster gene gun vaccinations 3 days apart. Separate batches of splenocytes were pulsed from the respective knockout mice with TRP-1-derived, K(b)-restricted peptide 455–463 TAPDNLGYM, TRP-2-derived, K(b)-restricted peptide 180–188 SVYDFFVWL or control HPV16E7-derived, D(b)-restricted peptide 49–57 RAHYNIVTF (Pi Proteomics, Huntsville, AL, USA). Then, cells were loaded with 1, 3, and 9 μM carboxyfluorescein succimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA), respectively. One and 9 μM concentrations were used where only two peptides were analyzed in the assay. Labeled cells were periorbitally injected 4 days after the final booster gene gun vaccination, and splenocytes were harvested after 18 h. Samples
representing 1000 irrelevantly pulsed splenocytes were acquired using FACS Canto equipment (BD Biosciences, Sparks, MD, USA) comparing the ratio of TRP-1 or TRP-2 peptide-pulsed splenocytes between vaccinated and naive mice from shifts in the area under the curves.

**Skin explant transfections and culture**

For ex vivo skin explant gene transfections, skin from two volunteer patients was biopsied and 1 mm × 2 mm skin pieces (three) were placed on each Statamatrix matrix (Cytomatrix Pty Ltd.; a gift from the laboratory of R. A. Clark, Brigham and Women’s Hospital, MA). In one experiment, on day 10, the skin/matrix was transfected (4.8-μg dose of total DNA) with wild-type or mutant (Q435A) HSP70i. On day 14, media were collected and cells were stained for HSP70i expression. For another experiment (as indicated), the skin/matrix was cultured in media only or with wild-type or mutant (Q435A) HSP70i protein (4 μg/ml). Half of the skin T media were refreshed every 2 to 3 days. On day 6, media were collected and cells were stained for transcription factors and homing markers.

**Flow cytometry**

For experiments including the Pmel-1 mice, splenocytes were recovered 25 weeks after the final vaccination and stained for the following surface markers: CD3, CD8, CD11b, CD11c, CLA, CXCR3, F4/80, and IgM-Thy1.2 (BD Biosciences). Initial gating was performed on live nondebris singlets, with subsequent gating toward CD11c+ versus CD11b+ cells using FACS LSR-II equipment (BD Biosciences). IgM+, Thy1.2+,
and CD3+ cells were excluded from the final gating. For intracellular profiles, antibodies to CD62L, T-bet, Eomes, FoxP3, CD44, and CD25 (eBioscience) were used. For experiments using in vitro-generated human DCs from PBMCs, the cells were gently spun and stained with mouse anti-human CD11c, CD80, CD83, CD86, and HLA-ABC antibodies labeled with allophycocyanin, phycoerythrin, phycoerythrin-cyan, fluorescein isothiocyanate, and V450 fluorochromes, respectively (BD Biosciences). Initial gating was performed on live nondebris singlets, with subsequent gating toward CD11c+ cells using FACSCanto II and FACS LSR-II equipment (BD Biosciences). For ImageStream X (Amnis) analysis, cells were incubated with primary antibodies SPA-820 toward HSP70i (Assay Designs) and 3D5 toward 6× His (histidine tag; Invitrogen) followed by phycoerythrin goat anti-mouse IgG1 (BD Biosciences) and allophycocyanin goat anti-mouse IgG2b (BD Biosciences) secondary antibodies, respectively.

**Statistical analyses**

All data are presented as means ± SEM, unless otherwise indicated. Distributions of data and means were compared with Wilcoxon’s rank sum test for depigmentation and immunohistochemistry data throughout, and *t* tests allowing for unequal variance for fluorocytometry, and Western blot data, except when skewness of the data suggested that the rank test would be more appropriate. Degrees of freedom for two-sample *t* tests were calculated with Satterthwaite’s formula. Two-sided tests were performed except for two data sets where we are confirming a previously
reported increase or decrease only (Western blot probed with SPA-810 and CXCR3 immunostaining of skin). Wilcoxon’s rank sum and \( t \) tests were calculated with Stata version 11 (StataCorp). Graphs were made with Prism software (GraphPad). \( P \) values less than 0.05 were considered statistically significant.


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VITA

The author, Jeffrey Mosenson is the son of Judy and Jerry Mosenson, and born in Elgin, IL on August 11, 1980. After graduating from Elgin High School in 1998, he obtained a B.S. at the University of Illinois at Chicago, graduating with highest distinction in the major of Biology. During summer breaks, Jeff worked as a pharmacy technician while enrolled at UIC. In May 2002, Jeff began work at Rush Medical Center as a patient care technician, assisting the nursing staff. In May 2003, Jeff joined the Cell Biology, Anatomy and Neurobiology M.S. program at Loyola University Chicago, where he worked in the lab of Dr. John McNulty. Jeff’s thesis project focused on characterizing the lymphocyte repertoire within the chicken pineal over a day/night cycle, which led to his first manuscript published in Biomed Central Immunology.

In August of 2005, Jeff began work as a research technician at Argonne National Laboratories where he modified techniques to isolate bacterial membrane proteins. In May 2008, Jeff joined the Cell Biology, Neurobiology, and Anatomy Ph.D. program at Loyola University Chicago. In June 2009, Jeff began work in the lab of Dr. Caroline Le Poole where he identified a central role for inducible heat shock protein 70 (HSP70i) in mediating autoimmune vitiligo. His work has also led to the generation of a mutant isoform of HSP70i which shows promise in treating vitiligo.
Jeff has won many travel awards to present his findings including the 21\textsuperscript{st} International Pigment Cell Conference in Bordeaux, France, the AAI Annual Meeting in Honolulu, Hawaii, and the IMTAC Interactive in Stockholm, Sweden. He was also invited to upload an online video based on his publication in *Pigment Cell and Melanoma Research*. In addition to several first author publications, Jeff’s research findings have received media attention which include articles published in the *Chicago Tribune*, and *Nature Immunology*.

Jeff will be pursuing a post-doctoral fellowship at the University of Illinois at Chicago in the lab of Dr. Wu, where he will study the development of stem cells, and utilize novel techniques such as genomic editing to treat diseases such as sickle cell anemia.